REMARKS

These remarks are in response to the Advisory Action dated April 9, 2004; the Office Action mailed September 22, 2003; and the October 20, 2004, interview with the Examiner. Claims 3 and 35-37 are pending in the application. No amendments to the claims have been made. Applicants request reconsideration and allowance of the pending claims. The specification has been amended to incorporate information from the "Brief Description of the Drawings" into the discussion of the drawings in Example 5. The phrase "and the NdeI 1.2 kb-#1 probe (from position 3709 to position 4337 of SEQ ID NO:3)" has been added to the description of Northern blot experiments at page 46, line 13, of the specification. Support for this amendment can be found in the "Brief Description of the Drawings" at page 27, line 6. The phrase "As shown in Figure 5" has been amended to "As shown in Figures 5 and 6" at page 46, line 27, of the specification, because a reference to Figure 6 was inadvertently omitted from the passage. No new matter has been added by this amendment.

Rejections Under 35 U.S.C. §§ 101/112, first paragraph

Applicants thank the Examiner for the gracious and helpful interview with the undersigned and her associate, Dr. Margo Furman, on October 20, 2004, concerning the rejection of claims 3 and 35-37 for lacking utility. During the interview, Applicants' representatives pointed out a number of utilities of the claimed polypeptides, including for generating antibodies that would be useful (a) for detecting liver cells or lung cancer cells and (b) for identifying compounds that agonize or antagonize binding of SEQ ID NO:1 or SEQ ID NO:2 to the serotonin receptor 5-HT_{2C}. The polypeptides themselves are also useful in methods for identifying compounds that agonize or antagonize their binding to the 5-HT_{2C} receptor. These utilities are discussed below.

Claims 3 and 36 are directed to substantially pure polypeptides that include SEQ ID NO:1. Claims 35 and 37 are directed to substantially pure polypeptides that include SEQ ID NO:2. These polypeptides possess multiple PDZ domains. The polypeptides bind via the PDZ domains to proteins having a hydrophobic amino acid region at their C-terminus. These binding

proteins, referred to as "PDZ domain-binding proteins", are primarily transmembrane proteins that play a role in signal transduction, and particularly in cell proliferation, neural transmission, apoptosis, and malignant conversion.

1. Use for generating antibodies to be employed in detecting liver cells and lung cancer tissues.

In support of Applicant's position that use of the claimed polypeptides for generating antibodies is specific, substantial and credible, Applicants discussed Example 12 of the USPTO Utility Guidelines Training Materials. Example 12 describes a hypothetical specification that discloses a novel protein, A, isolated from a cell membrane preparation. A is the binding partner for protein X, a protein with no identified function. Based solely on these facts, the hypothetical applicant characterizes isolated protein A as a receptor for protein X, discloses a method of making monoclonal antibodies that bind to the receptor, and claims "isolated receptor A". Performing the requisite utility analysis, Example 12 reasons that use of receptor A to make monoclonal antibodies that bind to receptor A qualifies as a "specific" utility for that receptor, thus meeting the "specific" aspect of the utility guidelines' criteria. Thus, it is clear that Applicants' proposed use of the claimed polypeptides to generate antibodies that bind to the claimed polypeptides should also qualify as sufficiently "specific".

Example 12 of the Training Materials goes on to state that the asserted utility is not, under the facts of that hypothetical case, "substantial." The basis for this conclusion lies in the fact that

> the only utility asserted for the identified materials is a therapeutic to effect control over receptor A. Since neither the specification nor the art of record disclose any diseases or conditions associated with receptor A, a method of treating an unspecified, undisclosed disease or condition, does not define a "real world" context of use.

Thus, if the Example 12 hypothetical specification had disclosed a specific disease or condition that could potentially be treated with antibodies specific for receptor A, then the requisite "real world" context of use would have been present and the "substantial" criterion met. Furthermore, Example 12 points out that, if the hypothetical specification had disclosed that receptor A is

present on the cell membranes of melanoma cells but not on the cell membranes of normal skin cells, the antibodies would have a specific and substantial, well established use in diagnosing melanoma. Such a use, according to the Training Materials, would be sufficient to meet the utility requirement.

In the present case, Applicants have disclosed that the gene(s) encoding the presently claimed polypeptides are highly expressed in certain tissues, including liver cells and lung tumor cells, and are expressed at low or undetectable levels in normal adult lung tissue. See Example 5 on pages 46-47 of the specification and Figure 6.[1] This evidence supports the utility of the antibodies for detection of either liver cells or lung cancer cells, consistent with the statements in Example 12 of the Training Materials. However, during the interview the Examiner noted that "Office policy" is to hold such evidence as insufficient to meet the utility requirement, maintaining that, according to "Office policy": (1) detection of normal liver cells is not a "specific and substantial utility", and (2) evidence that a cultured lung tumor cell line overexpresses a gene is not evidence that primary lung tumor tissue would also overexpress it (thus apparently challenging the "credibility" of the second utility, as opposed to its specificity or substantiality). The Examiner was unable to cite legal support for either of these apparently unwritten "policies" regarding the utility requirement, neither of which appears consistent with the Training Materials. Regarding the above "Office policy" (1), Applicants traverse the conclusion that use of the claimed polypeptides as markers for liver cells isn't "specific" or "substantial". As clearly set forth in Example 12 of the Training Materials (at page 65), use of a claimed polypeptide to produce an antibody meets the "specific" prong of the utility requirement. By the same logic, so would use of the antibody to bind its cognate antigen. Applicants have already provided arguments and evidence in the Replies filed on December 3, 2002, July 11, 2003, and March 19, 2004, that use of an antibody to identify a particular cell type is "substantial". "Substantial" merely means that it is a "real world" use, and use in discriminating between one cell type and another (whether liver vs. non-liver or lung cancer vs. normal lung) is

[1] Figure 6 was filed with the specification as Figure 5. This inadvertent error was corrected by amendment in the Transmittal of Formal Drawings mailed on November 8, 2000.

certainly "real world". Indeed, the Examiner has admitted in the Office Action mailed September 22, 2003, that liver cell markers are known in the art.[2]

Regarding the above "Office policy" (2) concerning the "credibility" of use in discriminating between lung tumor cells and normal adult lung cells, Applicants note that, according to the USPTO Utility Guidelines Training Materials, an assertion of utility is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion. The logic underlying the assertion that an antibody specific for the claimed polypeptides is useful for detection of lung tumor cells is neither "seriously flawed" nor "based on facts inconsistent with that logic". While it may be true that some gene expression is altered in cancer cell lines compared to primary cancer tissue, it is illogical to conclude that any and all observations with cancer cell lines are meaningless simply because expression "might" be different, particularly in the absence of any evidence to that effect. Cancer researchers often work with tumor cell lines as surrogates for primary cancer cells. Furthermore, Applicants note that overexpression of the gene of the invention was also observed in fetal lung tissue (p. 46, lines 29-30). Given that many genes overexpressed in cancers are also overexpressed in embryonic or fetal tissue, this lends further credence to Applicants' contention. The Examiner offers no evidence to contradict Applicants' evidence, such as a publication indicating that the gene of the invention is in fact not expressed to any substantial degree in any primary lung cancers. Rather, the Examiner simply asserts that "Office policy" is to treat evidence such as Applicants' as insufficient to establish a patentable utility, period.

Applicants respectfully request that the Examiner either withdraw her rejection of the claims for lack of utility, or provide concrete evidence (beyond a mere assertion of "Office policy") that the utilities proposed by Applicants are not credible.

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[2] The Office Action mailed September 22, 2003, cited the fact that liver cell markers are not novel as support for the utility rejection, as though the utility itself had to be a novel one. This of course is not the law.

2. Use in methods for identifying compounds that agonize or antagonize activity of 5-HT_{2C} receptors.

Applicants have asserted that the claimed polypeptides are involved, e.g., in neural transmission and are useful in methods for screening for proteins to which they bind. See, e.g., page 2, line 31, to page 3, line3, and page 5, lines 1-4, of the specification. In the interview, Applicants' representatives presented post-filing date evidence that the claimed polypeptides bind to 5-HT_{2C} receptors, receptors known to be involved in neural transmission. Ullmer et al.[3] ("Ullmer"; *FEBS Letters*, 424:63-68, 1998) isolated a cDNA encoding a 454 amino acid polypeptide fragment that is 98% identical to a portion of SEQ ID NO:1 and SEQ ID NO:2. Ullmer's cDNA was isolated in a two-hybrid screen for polypeptides that bind to the C-terminus of a serotonin receptor, the 5-HT_{2C} receptor (Ullmer, p. 65, left column). An alignment of a portion of SEQ ID NO:1 and the sequence of the fragment identified by Ullmer is shown in Figure 2 of the specification and the description of Figure 2 in the "Brief Description of the Drawings" in the specification at page 26, lines 14-15. As shown in the Figure 2, this portion of SEQ ID NO:1 (and SEQ ID NO:2) is identical to Ullmer's fragment at all but 7 amino acid residues. Ullmer also isolated cDNA encoding a full-length rat homolog of the partial human cDNA. He named the human and rat polypeptides MUPP1 for multi-PDZ-domain containing protein 1. Ullmer's fragment of the human MUPP1 polypeptide shares 96.6% identity to the corresponding portion of the rat MUPP1 sequence. Further studies have identified a specific PDZ domain, PDZ 10, of the full length rat sequence as the particular PDZ domain that is responsible for binding to 5-HT_{2C} receptors (see abstracts of Becamel et al., *J Biol Chem.*, 276(16):12974-12982, 2001; and Parker et al., *J Biol Chem.*, 278(24): 21576-21583, 2003; copies attached as Exhibits A and B, respectively). PDZ 10 of the full length rat sequence is identical to the corresponding region in the presently claimed SEQ ID NO:1 except for two amino acid changes: glutamine 1620 in the rat sequence is an arginine in position 939 of SEQ ID NO:1 (compare the sequence shown in Ullmer, Figure 1, p. 64, to SEQ ID NO:1); and the isoleucine at position 1638 of the rat sequence is a phenylalanine at position 957 of SEQ ID

[3] Ullmer et al. was cited in the Information Disclosure Statement filed by Applicants on May 10, 2000, and also is cited on page 2, line 16, of the specification.

NO:1. (Note that Ullmer's fragment of the human MUPPI sequence also contains this PDZ10 domain, with only one amino acid different from the corresponding domain of SEQ ID NO:1, at position 957 of SEQ ID NO:1.) Thus, the sequences are nearly identical within the particular PDZ domain that has been shown to bind 5-HT_{2C} receptors.

The utilities of the claimed polypeptides in methods for screening for compounds that agonize or antagonize their binding to 5-HT_{2C} receptors, or for generating antibodies to be used in the methods, is specific, substantial, and credible. The utilities are specific because they are specific to the subject matter claimed and are not applicable to all proteins, or even all PDZ-domain containing proteins. The utilities have a number of substantial "real world" applications: for example, screening for interaction of polypeptides comprising SEQ ID NO:1 or SEQ ID NO:2 with 5-HT_{2C} receptors can allow one to identify agents that modulate (e.g., increase or decrease) that interaction. Modulation of 5-HT_{2C} receptor activity is known to have pharmacological effects. For example, Clozapine, a drug used in treatment of schizophrenia, targets 5-HT_{2C} and 5-HT_{2A} receptors (see p. 432 of Stadel et al., *TiPS*, 18:430-436, 1997; copy attached as Exhibit C). Activation of 5-HT_{2C} receptors inhibits their ability to bind MUPP1, indicating that the interaction between the two polypeptides is involved in signaling of 5-HT_{2C} receptors (Parker, abstract). Thus, assays to identify agents that modulate the interaction would have applications in the treatment of disorders, such as schizophrenia, in which targeting serotonin receptors has a beneficial effect. Finally, the utilities are credible. As discussed above, an assertion is credible unless the logic underlying the assertion is seriously flawed, or unless the facts on which the assertion is based are inconsistent with the logic underlying the assertion. The present specification's assertion that the polypeptides of the invention would bind to receptor proteins involved in neural transmission is entirely consistent with the later studies on extremely closely related polypeptides and so certainly cannot be dismissed as not credible. Therefore, Applicants submit that these utilities are credible. Because all three elements required to establish utility are satisfied, withdrawal of the rejection under 35 U.S.C. §§ 101/112, first paragraph, is respectfully requested.

Enclosed is a a Request for Continued Examination and check for the Petition for Extension of Time fee. Please apply any other charges or credits to deposit account 06-1050, referencing Attorney Docket No. 14875-056001.

Respectfully submitted,

Date: 22 October 2004

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 16, Issue of April 20, pp. 12974–12982, 2001
Printed in U.S.A.

Interaction of Serotonin 5-Hydroxytryptamine Type 2C Receptors with PDZ10 of the Multi-PDZ Domain Protein MUPP1*

Received for publication, September 5, 2000, and in revised form, December 13, 2000
Published, JBC Papers in Press, January 9, 2001, DOI 10.1074/jbc.M008089200

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By using the yeast two-hybrid system, we previously isolated a cDNA clone encoding a novel member of the multivalent PDZ protein family called MUPP1 containing 13 PDZ domains. Here we report that the C terminus of the 5-hydroxytryptamine type 2C (5-HT_{2C}) receptor selectively interacts with the 10th PDZ domain of MUPP1. Mutations in the extreme C-terminal SSV sequence of the 5-HT_{2C} receptor confirmed that the SXV motif is critical for the interaction. Co-immunoprecipitations of MUPP1 and 5-HT_{2C} receptors from transfected COS-7 cells and from rat choroid plexus verified this interaction *in vivo*. Immunocytochemistry revealed an SXV motif-dependent co-clustering of both proteins in transfected COS-7 cells as well as a colocalization in rat choroid plexus. A 5-HT_{2C} receptor-dependent unmasking of a C-terminal vesicular stomatitis virus epitope of MUPP1 suggests that the interaction triggers a conformational change within the MUPP1 protein. Moreover, 5-HT_{2A} and 5-HT_{2B}, sharing the C-terminal EX(V/I)SXV sequence with 5-HT_{2C} receptors, also bind MUPP1 PDZ domains *in vitro*. The highest *MUPP1* mRNA levels were found in all cerebral cortical layers, the hippocampus, the granular layer of the dentate gyrus, as well as the choroid plexus, where 5-HT_{2C} receptors are highly enriched. We propose that MUPP1 may serve as a multivalent scaffold protein that selectively assembles and targets signaling complexes.

There is ample evidence suggesting that the function of a receptor is dependent on its specific subcellular localization. Sequence-specific interactions between proteins provide the basis for the structural and functional organization of receptors within cells. For a few members of the G-protein-coupled receptor family, these interactions have been described to be mediated by C-terminal interactions with PDZ (PSD-95/discs large/ZO-1) domain-containing proteins. The best investigated example is the β_2-adrenergic receptor, which interacts with the Na^+/H^+ exchanger regulatory factor (NHERF/EBP50)[1] (1). The interaction of NHERF with the β_2-adrenergic receptor is mediated via binding of the first PDZ domain of NHERF to the extreme C terminus of the β_2-adrenergic receptor in an agonist-dependent manner, thereby regulating Na^+/H^+ exchange (2). NHERF has also been described to link proteins with the actin cytoskeleton through association with ERM (ezrin-radixin-moesin) proteins (3). In fact, the PDZ-mediated interaction of the β_2-adrenergic receptor with NHERF family proteins has been shown to control recycling of internalized β_2-adrenergic receptors. Disrupting the β_2-adrenergic receptor-NHERF interaction perturbs the endocytic sorting of the β_2-adrenergic receptor, resulting in lysosomal degradation (4).

C-terminal interactions of G-protein-coupled receptors with PDZ domain-containing proteins extends to a member of the somatostatin receptor family (sst). The subtype sst2 interacts selectively with a highly homologous PDZ domain contained within the protein CortBP1/ProSAP1/Shank2 (5) and the recently cloned synaptic protein SSTRIP (somatostatin receptor-interacting protein)/Spank1/synamon/Shank1 (6). Both proteins belong to a common family recently termed Shanks (7, 8) or ProSAP (9), sharing essentially identical domain structures such as ankyrin repeats, an SH3 domain, the PDZ domain, a sterile α motif domain, and a proline-rich region that links Shanks to cortactin, a constituent of the actin cytoskeleton (10).

5-HT_{2C} receptors are broadly expressed in the central nervous system and in the choroid plexus (11, 12) and are involved in a diversity of physiological functions such as the control of nociception, motor behavior, endocrine secretion, thermoregulation, modulation of appetite, and the control of exchanges between the central nervous system and the cerebrospinal fluid (13–17). These receptors contain a C-terminal sequence, SSV* (where the asterisk indicates a carboxyl group), corresponding to the T/SXV* motif. This motif is potentially implicated in protein-protein interactions with PDZ domains as originally described for the C termini of the *N*-methyl-D-aspartate receptor and K^+ channel subunits (18, 19). The T/SXV* motif is also present in C termini of various other G-protein-coupled receptors, including the 5-HT_{2A} (18) and human 5-HT_{2B} receptors. By virtue of its interaction with the C terminus of the 5-HT_{2C} receptor, we recently isolated a novel cDNA encoding MUPP1, a protein with 13 PDZ domains (20). MUPP1 belongs to the family of multi-PDZ proteins comprising CIPP (channel-interacting PDZ domain protein) (21, 22), INADL (INAD-like protein) (23), and a putative *Caenorhabditis elegans* polypeptide referred to as C52A11.4 (20), containing 4, 7, or 10 PDZ do-

* This work was supported by grants from CNRS. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ Incumbent of the Madeleine Haas Russell Career Development Chair.

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[1] The abbreviations used are: NHERF, Na^+/H^+ exchanger regulatory factor; 5-HT_{2C}, 5-hydroxytryptamine type 2C; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; VSV, vesicular stomatitis virus; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid.

mains, respectively, and no other obvious catalytic domain. PDZ domains highly similar to those of MUPP1 are arrayed in the same order in all four proteins, implying the requirement of a precise arrangement for the assembly into a functional macromolecular complex (20). The present work provides biochemical and immunohistochemical evidence that the 5-HT$_{2C}$ receptor interaction with MUPP1 takes place *in vitro* and *in vivo*. Among the 13 PDZ domains of MUPP1, 5-HT$_{2C}$ receptors exclusively interact with the 10th PDZ domain, emphasizing the high selectivity of PDZ domain interactions. Moreover, the interaction induces a conformational change in the MUPP1 molecule and a co-clustering that might trigger a downstream signal transduction pathway.

EXPERIMENTAL PROCEDURES

Two-hybrid Screening—Yeast two-hybrid screening was performed using the CG1945 strain (24) harboring the *HIS3* and β-galactosidase reporter genes under the control of upstream *GAL4*-binding sites (CLONTECH). The yeast culture was transformed using the polyethylene glycol/LiAc method (25). Interactions between bait and prey were monitored by β-galactosidase activity in colonies transferred onto Hybond N filters (Amersham Pharmacia Biotech, Freiburg, Germany).

Domain Analysis of the Interaction—To assemble the full-length clone pBSKSII-rMUPP1, a PCR fragment covering region −183 to 2000 with a silent mutation of C^{1979} to T to delete a *Bam*HI site was generated by reverse transcription-PCR from rat femoral muscle cDNA that was prepared as described (26) using recombinant *Pfu* polymerase (Stratagene, La Jolla, CA). The resulting 5′-end was ligated into the remaining *Bam*HI site of the partial rat *MUPP1* cDNA (pXMD1/rMUPP1) (20). pBSKSII-rMUPP1 was sonicated to an average size of 500 base pairs, and blunt-ended DNA fragments were cloned into the *Srf*I site of pCR-Script (Stratagene). The inserts of 20,000 recombinant plasmids retrieved by *Not*I and *Eco*RI digestion were directionally cloned into the *Xho*I and *Eco*RI sites of pACT-2 using *Not*I/*Xho*I adapter oligonucleotides. The resulting library was transformed into yeast strain CG1945 carrying pAS2-1/hu2C. pAS2-1/hu2C was described previously (20). The resulting plasmid fuses amino acids 369–458 of human 5-HT$_{2C}$ receptors to the Gal4 DNA-binding domain (amino acids 1–147) of yeast Gal4 in the yeast expression vector pAS2-1 (CLONTECH). Transformants selected for *HIS3* expression were picked after 5 days. Plasmid DNA was extracted, and inserts were retrieved by PCR with plasmid-specific primers, sequenced, and aligned with the rat *MUPP1* cDNA sequence.

Mutagenesis—The 90-amino acid C-terminal fragment of the human 5-HT$_{2C}$ receptor was mutagenized by PCR using reverse primers harboring nucleotide exchanges and by using pAS2-1/hu2C as template. The amplified DNA was directionally subcloned into the *Bam*HI and *Eco*RI sites of pBluescript KSII (Stratagene) and sequenced. The inserts were transferred into either pAS2-1 (*Bam*HI and *Sal*I) or pGEX-3X (*Bam*HI and *Eco*RI; Amersham Pharmacia Biotech). The mutant plasmid pRK5/h5-HT$_{2C\text{-}SSA}$ was obtained by PCR using a reverse primer encoding the V458A mutation by a GCG codon using pRK5/h5-HT$_{2C}$ as template.

In Vitro Protein-Protein Interaction—The partial cDNA of human MUPP1 was amplified by PCR from pACT-2/huMUPP1 (clone 1) (20) and inserted directionally into the *Hin*dIII and *Eag*I sites of pBAT (27). *In vitro* translation of [^{35}S]methionine-labeled MUPP1 was performed using T3 RNA polymerase with the TNT coupled reticulolysate lysate system (Promega, Mannheim, Germany) according to the manufacturer's instructions. The C-terminal tails of the 5-HT$_{2A}$ (residues 380–471), 5-HT$_{2B}$ (residues 414–481), and 5-HT$_{2C}$ (residues 368–458) receptors were subcloned into pGEX-3X, giving rise to plasmids pGEX2A92, pGEX2B67 and pGEX2C90, respectively. Synthesis of recombinant proteins (GST-2A92 and GST-2C90) in BL21 cells (Amersham Pharmacia Biotech) was induced by 0.25 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30 °C. GST-2B67 was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 25 °C. Cells were sonicated in buffer S (20 mM HEPES (pH 7.9), 100 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and pelleted in buffer S, 1% Triton, and 10% glycerol. GST proteins were purified on bulk glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions, with the exception of using buffer S instead of PBS. GST proteins were eluted with buffer S, 10% glycerol, and 10 mM glutathione and dialyzed against buffer S and 10% glycerol (2 × 3 h at 4 °C). ^{35}S-Labeled MUPP1 was incubated in buffer S, 10% glycerol, and 1% Nonidet P-40 with glutathione-Sepharose 4B beads saturated with 10 μg of GST fusion protein or GST for 3.5 h at 4 °C in the presence or absence of 1.5 mM synthetic peptide VVSERISSV or control peptide VVSERIASA (B&G Biotech GmbH, Freiburg). Beads were washed four times for 10 min in buffer S, 10% glycerol, and 1% Nonidet P-40. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Expression Plasmids—pBSKSII-rMUPP1 was cut with *Eco*RI and *Xho*I and ligated with a PCR product containing a C-terminal VSV tag flanked by two *Xho*I sites into pXMD1 (28), generating pXMD1/rMUPP-VSV. pBSKSII-rMUPP1 was cut with *Nde*I and *Sal*I and ligated into pCI-neo (Promega) *Eco*RI and *Sal*I sites using adapter oligonucleotides containing the VSV tag (YTDIEMNRLGK) DNA sequence (TACACCGATATCGAGATGAACAGGCTGGGAAAGTGA). The resulting plasmid, pCI-neo/VSV-ΔMUPP1, fuses the N-terminal VSV tag and amino acids 1337–2055 of the rat MUPP1 protein. pRK5/h5-HT$_{2C}$ was directionally subcloned from pXMD1/h5-HT$_{2C}$ (29) by releasing the cDNA insert with the restriction digest enzymes *Eco*RI and *Xba*I. Using PCR, the human 5-HT$_{2C}$ receptor cDNA was N-terminally tagged with a stretch of nucleotides (ATGGAACAAAAGCTTATTTCTGAAGAAGACTTG) encoding a 10-amino acid epitope (EQKLISEEDL) of the human c-Myc protein (30). The amplified DNA was directionally subcloned into the *Eco*RI and *Xba*I sites of pRK5, yielding pRK5/c-Myc-hu5-HT$_{2C}$.

Cell Culture and Transfection—Expression plasmids were introduced into COS-7 cells by electroporation as described (31). Briefly, cells were trypsinized, centrifuged, and resuspended in electroporation buffer (50 mM K_2HPO_4, 20 mM CH_3CO_2K, 20 mM KOH, and 26.7 mM $MgSO_4$ (pH 7.4)) with 1 μg of pRK5/h5-HT$_{2C}$ and 1.5 μg of pXMD1/rMUPP-VSV. The total amount of DNA was kept constant at 15 μg by filling up with pRK5 DNA. After 15 min at room temperature, 10^7 cells were transferred to a 0.4-cm electroporation cuvette (Bio-Rad) and pulsed using a Gene Pulser apparatus (setting at 1000 microfarads and 280 V). Cells were resuspended in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum (Life Technologies, Inc.) and plated on 10-cm Falcon Petri dishes or into 12-well clusters.

Antibodies—The production, characterization, and purification of the rabbit polyclonal 522 antibody raised against the mouse 5-HT$_{2C}$ receptor have been described (32). The mouse monoclonal anti-c-Myc antibody was a gift from B. Mouillac. Rabbit polyclonal anti-MUPP1 antiserum was raised against GST fusion protein containing the third PDZ domain of rat MUPP1 (residues 318–451) (antibody 2324) or amino acids 780–1063 of human MUPP1, containing the region between the fourth and fifth PDZ domains (antibody 2526).[2] The mouse monoclonal anti-VSV antibody was purchased from Sigma. The secondary antibodies used were Oregon green-conjugated goat anti-rabbit or anti-mouse antibody and Texas Red-conjugated goat anti-mouse or anti-rabbit IgG antibody (all from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Membrane Preparations and Immunoprecipitation—Rat choroid plexus or COS-7 cells were briefly centrifuged (3 min at 200 × *g*), and pellets were resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, and protease inhibitor mixture (2.5 μg/ml each leupeptin, aprotinin, and antipain and 0.5 mM benzamidine (pH 7.4))), homogenized 20 times with a glass-Teflon homogenizer at 4 °C, and centrifuged at 100,000 × *g* for 1 h. Each membrane pellet was resuspended in CHAPS extraction buffer (50 mM Tris-HCl (pH 7.4) containing 0.05 mM EDTA, 10 mM CHAPS, and protease inhibitor mixture (see above)) for 2 h in rotation at 4 °C. After centrifugation (1 h at 100,000 × *g*), CHAPS-soluble proteins were incubated overnight at 4 °C with 2 μl of anti-5-HT$_{2C}$ receptor 522 antibody, anti-VSV antibody, or anti-MUPP1 antiserum for the immunoprecipitation. 50 μl of protein A-Sepharose beads (Sigma) was added to the supernatant, and the mixture was then rotated at 4 °C for 1 h. After five washes in CHAPS-free buffer, the immunoprecipitates and co-immunoprecipitates were dissociated in Laemmli sample buffer. The samples were centrifuged, and the supernatants were fractionated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel, electrotransferred to nitrocellulose membrane (Hybond C extra, Amersham Pharmacia Biotech), probed with rabbit anti-5-HT$_{2C}$ receptor antibody or anti-MUPP1 antiserum (1:500), and then detected by enhanced the chemiluminescence method (Renaissance Plus, PerkinElmer Life Sciences).

Determination of Inositol Phosphate Accumulation—6 h after transfection, cells were incubated overnight in serum-free Dulbecco's modified Eagle's medium with 1 μCi/ml *myo*-[^{3}H]inositol (17 Ci/mol; PerkinElmer Life Sciences). Total inositol phosphate accumulation in

[2] S. Poliak and E. Peles, manuscript in preparation.

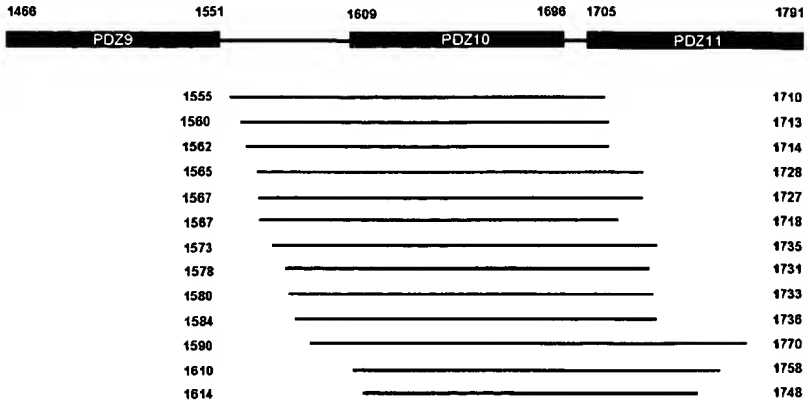


FIG. 1. **The C-terminal domain of the 5-HT$_{2C}$ receptor interacts with PDZ10 of MUPP1.** Randomly generated MUPP1 fragments interacting in the yeast two-hybrid system with the C-terminal sequence of the 5-HT$_{2C}$ receptor are displayed relative to the domain map and are identified by amino acid numbers. All selected clones share the PDZ10 coding sequence (*shaded*).

response to serotonin in the presence of 10 mM LiCl for 10 min at 37 °C was determined as described (33). The amount of [^{3}H]inositol phosphate formed was separated according to the ion exchange method (34). Concentration-response curves, EC_{50} values, and Scatchard analysis were calculated with Synergy software.

Membrane Preparations and Radioligand Binding Assay—Membranes were prepared from transiently transfected COS-7 cells. 6 h after transfection, cells were incubated overnight in serum-free Dulbecco's modified Eagle's medium. Cells were washed twice in PBS, scraped with a rubber policeman, harvested in PBS, and centrifuged at 4 °C for 4 min at 200 × *g*. The pellet was resuspended in 10 mM HEPES (pH 7.4), 5 mM EGTA, 1 mM EDTA, and 0.32 M sucrose and homogenized 10 times with a glass-Teflon homogenizer at 4 °C. The homogenate was centrifuged at 100,000 × *g* for 20 min, and the membrane pellet was resuspended in 50 mM HEPES (pH 7.4) and stored at −80 °C until used. 5-HT$_{2C}$ receptor densities were estimated using the specific radioligand [*N*^{6}-*methyl*-^{3}H]mesulergine at a saturating concentration of 4 nM. Mianserin (1 μM) was used to determine nonspecific binding. Protein concentrations were determined using the Bradford protein assay (Bio-Rad).

Immunocytochemistry—Cells were grown on 35-mm dishes, fixed 24 h after transfection in 4% paraformaldehyde and PBS (pH 7.4) for 20 min at room temperature, washed three times in 0.1 M glycine buffer (pH 7.4), and permeabilized with 0.05% Triton X-100 for 5 min. Cells were washed in 0.2% gelatin and PBS and incubated overnight at 4 °C with the primary antibody diluted 1:500 in 0.2% gelatin and PBS. Cells were washed and incubated for 1 h at room temperature with the secondary antibody diluted 1:1000 in 0.2% gelatin and PBS. Cultures were washed, mounted on glass slides using Gel Mount (Biomeda Corp., Foster City, CA), and viewed on a Zeiss Axioplan 2 microscope (Zeiss, Göttingen, Germany).

Enzyme-linked Immunosorbent Assay—Cells were seeded at a density of 8 × 10^5 cells/well on a 12-well plate, fixed 24 h after transfection in 4% paraformaldehyde and PBS (pH 7.4) for 20 min at 4 °C, and washed three times in 0.1 M glycine buffer (pH 7.4). Cells were incubated for 5 min in 3% H_2O_2 and PBS to minimize endogenous peroxidase activity. The anti-c-Myc antibody (diluted 1:500 in PBS and 0.2% bovine serum albumin) was applied for 1.5 h at 37 °C. Plates were rinsed five times with PBS and incubated for 1 h at 37 °C with horseradish peroxidase-conjugated anti-mouse antibody diluted 1:250 in PBS and 0.2% bovine serum albumin. Plates were rinsed seven times with PBS, and the reaction was developed by adding the substrate ABTS. Absorbance was measured at 410 nm using an enzyme-linked immunosorbent assay reader. Control plates without cells were included to determine background activity, which was subtracted from the A_{410} readings. Each experiment was performed in quadruplicates.

Immunohistochemistry—Rats were deeply anesthetized with pentobarbital and transcardially perfused with a fixative solution containing 4% paraformaldehyde and 0.1 M PBS (pH 7.4). Brains were removed, post-fixed at 4 °C for 2 h in the same fixative solution, and stored overnight at 4 °C in PBS containing 30% sucrose for cryoprotection. Sections of 10 μm were cut with a cryostat (Microm HM500), collected on slides, and then stored at −80 °C. Prior to the experiment, sections were rinsed serially for 5 min once with PBS containing 20% sucrose, 10% sucrose, and without sucrose, respectively. Preincubation was performed in 0.25% Triton X-100, 20% horse serum, and PBS (pH 7.4) for 1 h at room temperature. Sections were incubated with either the rabbit polyclonal anti-5-HT$_{2C}$ receptor 522 antibody or affinity-purified anti-MUPP1 2324 or 2526 antibody diluted 1:500 in 1% horse serum and PBS overnight at 4 °C. After three washes in PBS, sections were incubated with donkey Cy3-conjugated anti-rabbit IgG antibody (1:2000; Jackson ImmunoResearch Laboratories, Inc.) in 1% horse serum and PBS at 4 °C for 3 h. Sections were rinsed with PBS and mounted on slides, which were coverslipped with Mowiol (Calbiochem).

In Situ Hybridization—Rats (10-week-old Wistar male adult) were killed by decapitation, and their brains were removed and frozen immediately on dry ice. Microtome cryostat sections (20 μm) were thaw-mounted onto gelatin-coated slides, air-dried, and kept at −20 °C until used. Hybridization was performed at 60 °C essentially as described (35) using radioactive [α-^{33}P]UTP-labeled riboprobes that were prepared as follows. The plasmid pXMD1/rMUPP1 (20) was cut with *Sma*I to release a fragment of 2669 base pairs covering the region from 4653 to the 3′-end of the rat *MUPP1* cDNA, which was ligated into the *Srf*I site of pCR-Script. For *in vitro* transcription, both sense and antisense constructs were linearized with *Xho*I and transcribed using T3 RNA polymerase. RNA synthesis was performed in a 20-μl reaction with 1 μg of linearized plasmid DNA, 10 mM dithiothreitol, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.1 mM UTP, 70 μCi of [α-^{33}P]UTP, 40 units of RNasin (Promega), and 20 units of RNA polymerase (Roche Molecular Biochemicals, Mannheim) and incubated for 1 h at 37 °C. Synthesis was continued with the addition of 20 units of RNasin and 20 units of RNA polymerase for 1 h. DNA was digested with 10 units of RQ1 RNase-free DNase (Promega) for 20 min. The reaction was stopped with 5 μl of EDTA (0.5 M) and applied to a Sephadex G-50 spin column (Roche Molecular Biochemicals). RNA was hydrolyzed in 20 mM $NaHCO_3$ and 30 mM $NaCO_3$ for 20 min and neutralized in 3.3 mM HCl.

RESULTS

Characterization of the PDZ Domain Interaction—It is evident that PDZ domains display sufficient variability to allow distinct protein-protein interactions (36, 37). Since rat MUPP1 has 13 PDZ domains, we analyzed the interaction of all parts of MUPP1 with the C terminus of the 5-HT$_{2C}$ receptor. A library of fusion proteins with the Gal4 activation domain was constructed through a random generation of ~500-base pair DNA fragments by sonication of pBSKSII-rMUPP1. This tagged fragment library was cotransfected into yeast with the vector encoding the C terminus of the 5-HT$_{2C}$ receptor attached to the Gal4 DNA-binding domain, and resultant yeast colonies were selected by histidine starvation. DNAs from 13 selected colonies were amplified by PCR with plasmid-specific primers, and the amplified DNAs were sequenced. All sequences shared the entire MUPP1 PDZ10 coding region (Fig. 1), indicating a selective interaction of the 5-HT$_{2C}$ receptor C terminus with PDZ10. The complete PDZ10 domain seems to be required since PDZ10 was complete in all selected clones.

Characterization of the 5-HT$_{2C}$ Receptor C-terminal Interaction—Ion channels such as the *N*-methyl-D-aspartate and Shaker-type K^+ channels share a C-terminal T/SXV* motif known to interact with PDZ domains (18, 19). The 5-HT$_{2C}$ receptor has a similar C-terminal SSV* sequence that may define a part of the critical motif for interaction. To determine whether the 3 C-terminal amino acid residues in the 5-HT$_{2C}$ receptor are essential for binding to the PDZ10 domain of MUPP1, mutational analysis was performed in which the res-

TABLE I
Sequence requirements in the 5-HT$_{2C}$ receptor carboxyl terminus mediating interaction with MUPP1

Single amino acid substitutions are shown in boldface. The ability to interact with MUPP1 was tested in the yeast two-hybrid assay. *HIS3* activity was measured by the percentage of colonies growing on histidine-lacking medium: ++, >60%; +, 30–60%; −, no significant growth. β-Galactosidase (β-Gal) activity was determined from the time taken for colonies to turn blue in the 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) filter lift assay: ++, <3 h; +, 3–6 h; −, no significant β-galactosidase activity.

5-HT$_{2C}$ carboxyl terminus (369–458)	*HIS3*	β-Gal
369-SSV (wild-type)	++	++
369-TSV	+	++
369-ASV	−	−
369-SAV	+	++
369-SSA	−	−

idue at each position was replaced with an alanine, and interactions with MUPP1 were tested in the yeast two-hybrid assay. A partial human MUPP1 clone (clone 1) (see Ref. 20) encoding the C-terminal 454 amino acids including PDZ10–13 served as bait. Substitution of Ser^{456} and Val^{458} with Ala abolished the interaction with MUPP1 (Table I). In contrast, the conservative mutation of Ser^{456} to Thr and the mutation of Ser^{457} to Ala were tolerated, although the resultant yeast clones displayed reduced growth on histidine-lacking medium. To independently demonstrate the interaction of MUPP1 with the 5-HT$_{2C}$ receptor C terminus, fusion proteins of either the wild-type form or various mutants of C-terminal 5-HT$_{2C}$ receptor sequences (90 amino acids) fused to GST were bound to glutathione-Sepharose (Fig. 2*A*) and incubated with *in vitro* translated ^{35}S-labeled MUPP1 (clone 1, PDZ10–13). MUPP1 bound to the GST-5-HT$_{2C}$ fusion proteins was resolved on an SDS-polyacrylamide gel and visualized by autoradiography. Only the S457A mutant (Fig. 2*A*, *fifth lane*) retained MUPP1-binding activity, as observed in the yeast two-hybrid assay (Table I), which confirmed the SXV motif as a critical determinant for the PDZ domain interaction.

Interaction of MUPP1 with 5-HT$_{2A}$ and 5-HT$_{2B}$ Receptors—Multiple sequence alignment of the C-terminal ends from all members of the 5-HT$_2$ receptor family (Fig. 2*B*) revealed a common C-terminal amino acid sequence motif, EX(V/I)SXV*. We therefore analyzed whether the 5-HT$_{2A}$ or 5-HT$_{2B}$ receptor C terminus would also interact with MUPP1. GST fusion proteins of the C-terminal 92 amino acids of the 5-HT$_{2A}$ receptor (GST-2A92) and the C-terminal 67 amino acids of the 5-HT$_{2B}$ receptor (GST-2B67) were bound to glutathione-coupled Sepharose beads and incubated with *in vitro* translated ^{35}S-labeled MUPP1 (clone 1, PDZ10–13). MUPP1 bound to both the GST-2A92 (Fig. 2*C*, *second lane*) and GST-2B67 (*fourth lane*) fusion proteins, but not to GST alone (*first lane*). Specificity of the interaction with MUPP1 PDZ domains was demonstrated by co-incubation with a 9-amino acid synthetic peptide mimicking the 5-HT$_{2C}$ receptor C terminus, which prevented MUPP1 binding to all three GST-5-HT$_2$ receptor fusion proteins (Fig. 2*C*, *third*, *fifth*, and *seventh lanes*). A control peptide harboring the S456A and V458A mutations did not compete with MUPP1 binding (data not shown), confirming the requirement of Ser (position −2) and Val (position 0) for PDZ domain interaction.

Interaction of Heterologously Expressed 5-HT$_{2C}$ Receptors with MUPP1—To determine whether MUPP1 forms a protein complex with the 5-HT$_{2C}$ receptor in living cells, COS-7 cells were transfected with cDNA encoding the human 5-HT$_{2C}$ receptor in the presence or absence of the C-terminally VSV-tagged rat MUPP1 protein (MUPP1-VSV). In a CHAPS-soluble cell extract, the anti-5-HT$_{2C}$ receptor 522 antibody (11) revealed two bands between 60 and 50 kDa, corresponding to the glycosylated and unglycosylated 5-HT$_{2C}$ receptor, respectively (Fig. 3 *first* and *fifth lanes*). In fact, treatment with *N*-glycosidase F to remove *N*-linked sugars caused a shift of the upper band to the level of the lower band (data not shown). CHAPS-soluble extracts were immunoprecipitated by an anti-VSV antibody and then immunoblotted with the anti-5-HT$_{2C}$ receptor 522 antibody. 5-HT$_{2C}$ receptors were co-immunoprecipitated by MUPP1-VSV from cells cotransfected with 5-HT$_{2C}$ receptor and MUPP1 expression plasmids (Fig. 3, *second lane*). This indicates that the MUPP1 protein and the 5-HT$_{2C}$ receptor are able to interact when expressed in a heterologous system. In contrast, when cells were transfected with the 5-HT$_{2C}$ receptor alone, the receptor was not revealed in the immunoprecipitates (Fig. 3, *sixth lane*), indicating the specificity of the co-immunoprecipitation. The mutant 5-HT$_{2C}$ receptor construct in which the C-terminal Val of the SXV PDZ-binding motif was replaced with Ala (V458A, 5-HT$_{2C\text{-}SSA}$) was extracted in amounts similar to those of the native 5-HT$_{2C}$ receptor, but to a higher extent in the glycosylated form (Fig. 3, *third lane*). When cells expressed MUPP1-VSV and the 5-HT$_{2C\text{-}SSA}$ receptor, the mutant receptor could not be immunoprecipitated with the anti-VSV antibody (Fig. 3, *fourth lane*). This is in accordance with the *in*

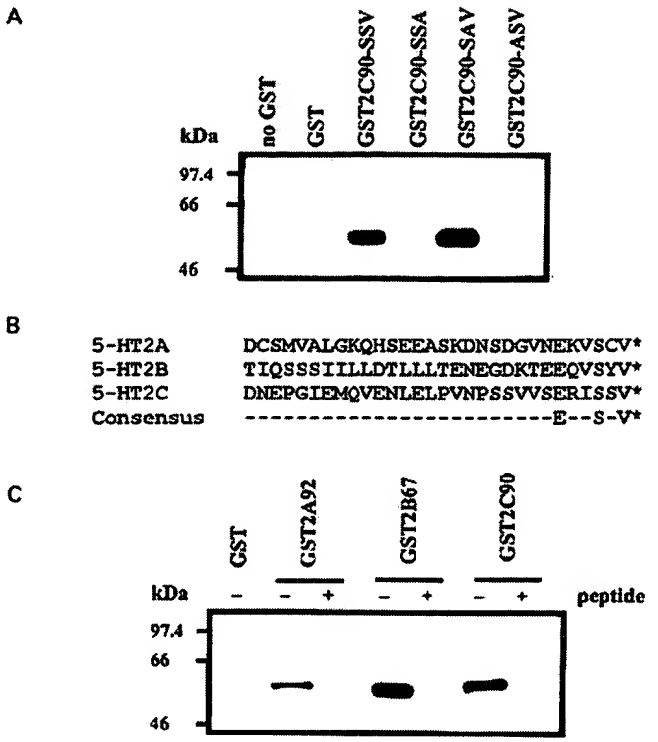


FIG. 2. ***In vitro* binding of C-terminal receptor sequences to MUPP1.** DNA encoding C-terminal receptor sequences in-frame with the GST moiety were bacterially expressed and purified on glutathione-Sepharose. GST pull-down reactions were performed with *in vitro* translated ^{35}S-labeled MUPP1 (clone 1, PDZ10–13), and the adsorbed proteins were separated by SDS-polyacrylamide gel electrophoresis. MUPP1 that was copurified with the fusion protein was identified by autoradiography. *A*, analysis of GST fusion proteins that contained 90-amino acid C-terminal 5-HT$_{2C}$ receptor sequences from either the wild-type form (GST-2C90-SSV) or mutants in which 1 of the last 3 amino acids was replaced by alanine as indicated. *B*, alignment of the C-terminal 30 amino acids of human 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. All sequences share the C-terminal EX(V/I)SXV* motif, where the asterisk indicates a carboxyl group. *C*, analysis of the GST fusion protein with the C-terminal 92 amino acids of the 5-HT$_{2A}$ receptor sequence (GST-2A92) or the C-terminal 67 amino acids of the 5-HT$_{2B}$ receptor (GST-2B67) or GST-2C90. Where indicated, an 8.5-fold excess of the EX(V/I)SXV sequence-containing peptide was present during incubation.

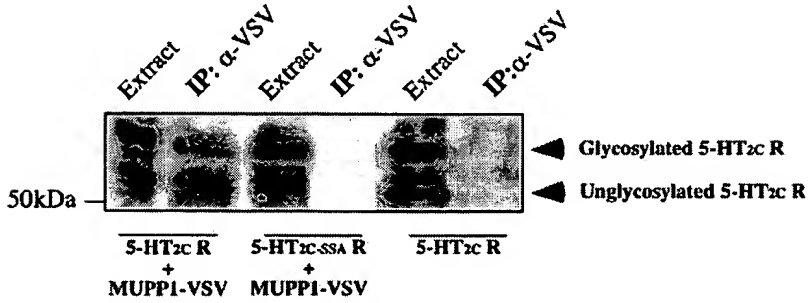


FIG. 3. **Co-immunoprecipitation of MUPP1 and 5-HT$_{2C}$ receptors from transfected COS-7 cells.** Cells were transfected with MUPP1-VSV and the 5-HT$_{2C}$ receptor (*5-HT2C R*) (*first* and *second lanes*), MUPP1-VSV and the mutant 5-HT$_{2C\text{-}SSA}$ receptor (*third* and *fourth lanes*), or the 5-HT$_{2C}$ receptor alone (*fifth and sixth lanes*). Membrane fractions were solubilized with CHAPS and then immunoprecipitated (*IP*) using the anti-VSV antibody (*second*, *fourth*, and *sixth lanes*). The immunoprecipitates were analyzed by Western blotting using the anti-5-HT$_{2C}$ receptor antiserum.

vitro experiments, confirming that the SXV motif at the extreme C terminus of the 5-HT$_{2C}$ receptor is the critical determinant for the interaction with MUPP1.

MUPP1 Induces 5-HT$_{2C}$ Receptor Clustering in COS-7 Cells—Because the antibodies to both MUPP1 and 5-HT$_{2C}$ receptors were derived from rabbits, a c-Myc epitope-tagged version of the human 5-HT$_{2C}$ receptor was constructed. Immunofluorescence was performed with COS-7 cells transiently expressing the *N*-terminally c-Myc-tagged 5-HT$_{2C}$ receptor. Staining with an anti-c-Myc antibody revealed a random distribution of 5-HT$_{2C}$ receptors on membrane-type structures including intracellular membranes similar to the distribution described previously (11) and in neurons (12) (Fig. 4*A*). In contrast, the MUPP1-VSV protein, which was stained with an antibody raised against the PDZ3 domain of MUPP1, was homogeneously distributed throughout transiently transfected COS-7 cells (Fig. 4*B*). The specificity of the anti-MUPP1 antibody was confirmed using the following criteria: the preimmune serum did not show any labeling (data not shown); preincubation with an excess (80 μg/ml) of a peptide (GST-PDZ3) corresponding to the PDZ3 domain suppressed the immunofluorescent staining (data not shown); and a single protein with the expected molecular mass of 230 kDa was revealed on Western blots prepared from total extracts of MUPP1-expressing COS-7 cells (data not shown) or from rat choroid plexus (see Fig. 8). Coexpression of the c-Myc-tagged 5-HT$_{2C}$ receptor together with MUPP1-VSV revealed a distribution that was distinct from those observed for each of them. Both proteins were colocalized and formed many clusters (Fig. 4*C*). A similar pattern of immunoreactive receptors was obtained when the c-Myc-tagged 5-HT$_{2C}$ receptor was coexpressed with a truncated form of the rat MUPP1 protein (VSV-ΔMUPP1) containing only the last six of the 13 PDZ domains (PDZ8–13) (Fig. 4*D*). When cells coexpressing MUPP1 and 5-HT$_{2C}$ receptors were treated with 5-hydroxytryptamine (30 min, 1 h, and overnight), no changes in the distribution of 5-HT$_{2C}$ receptors and MUPP1 were observed (data not shown).

Confocal microscopy revealed that the clustered complex of MUPP1 and 5-HT$_{2C}$ receptors was localized on intracellular membranes, but also on the cell surface (Fig. 4*E*). To further investigate cell-surface localization of the clustered complex, selective permeabilization was applied. In non-permeabilized cells, cell-surface expression of the N-terminally c-Myc-tagged 5-HT$_{2C}$ receptor could be immunostained by the anti-c-Myc antibody. When tagged 5-HT$_{2C}$ receptors were expressed alone, no significant labeling could be detected (data not shown). However, when cells coexpressed tagged receptors and MUPP1, few clusters were observed at the cell surface of non-permeabilized cells (Fig. 4*F*, *left panel*). This difference could be due to a better visualization of clustered *versus* dispersed receptors. Subsequently, the same cells were permeabilized and incubated with the anti-MUPP1 antibody, which stained numerous clusters (Fig. 4*F*, *center panel*). As expected, only those clusters localized on cell membranes were colocalized with 5-HT$_{2C}$ receptors (Fig. 4*F*, *right panel*). To investigate whether MUPP1 modulates cell-surface expression of 5-HT$_{2C}$ receptors, a cell-surface enzyme-linked immunosorbent assay was performed using non-permeabilized cells. Immunolabeled c-Myc-tagged 5-HT$_{2C}$ receptors were quantified by a secondary horseradish peroxidase-conjugated anti-mouse antibody. When c-Myc-tagged 5-HT$_{2C}$ receptors were transfected alone, the enzymatic activity was 223.45 ± 2.31% of the control values (mock-transfected cells), whereas the values were 212.95 ± 5.45% of the control values when cotransfected with MUPP1. This suggests that MUPP1 does not alter cell-surface expression of 5-HT$_{2C}$ receptors. In conclusion, these results indicate that MUPP1 induces clustering of a few 5-HT$_{2C}$ receptors at the cell surface, but the total number of cell-surface receptors remains unchanged. To test if this clustering is mediated by the C-terminal interaction of the 5-HT$_{2C}$ receptor with MUPP1, the mutant 5-HT$_{2C\text{-}SSA}$ receptor was coexpressed with MUPP1. Whereas the mutant receptor displayed a distribution similar to that of the wild-type 5-HT$_{2C}$ receptor in transfected COS-7 cells (data not shown), coexpression failed to form clusters (Fig. 4*G*).

5-HT$_{2C}$ Receptors Induce a Conformational Change in MUPP1—When MUPP1-VSV was transiently expressed in COS-7 cells, the anti-VSV antibody was unable to detect the MUPP1-VSV protein (Fig. 5*A*). However, when MUPP1-VSV was coexpressed with the 5-HT$_{2C}$ receptor, the same anti-VSV antibody succeeded in staining the MUPP1 protein (Fig. 5*B*). This clearly demonstrates that the VSV tag fused to the C terminus of the MUPP1 molecule is unmasked upon interaction of MUPP1 with the 5-HT$_{2C}$ receptor C terminus. This interaction may induce a conformational change and render the VSV tag accessible to the anti-VSV antibody.

Spatial Distribution of MUPP1 Transcripts in Rat Brain and Colocalization of MUPP1-5-HT$_{2C}$ Receptor Proteins in the Choroid Plexus—To verify that the MUPP1-5-HT$_{2C}$ receptor interaction does exist within a tissue that endogenously expresses both proteins, we first studied the spatial distribution of *MUPP1* transcripts in brain, where the distribution of 5-HT$_{2C}$ receptors is well known (11, 12). *In situ* hybridization studies were carried out using an [α-^{33}P]UTP-labeled riboprobe (Fig. 6). *MUPP1* transcripts are abundant in all cerebral cortical layers, especially the piriform cortex, the pyramidal cells of the CA1–CA3 subfields of the hippocampus, as well as the granular layer of the dentate gyrus. *MUPP1* mRNA was detected in the internal granular layer and the mitral cell layer of the olfactory bulb; in the medial habenular nucleus; and in amygdaloid,

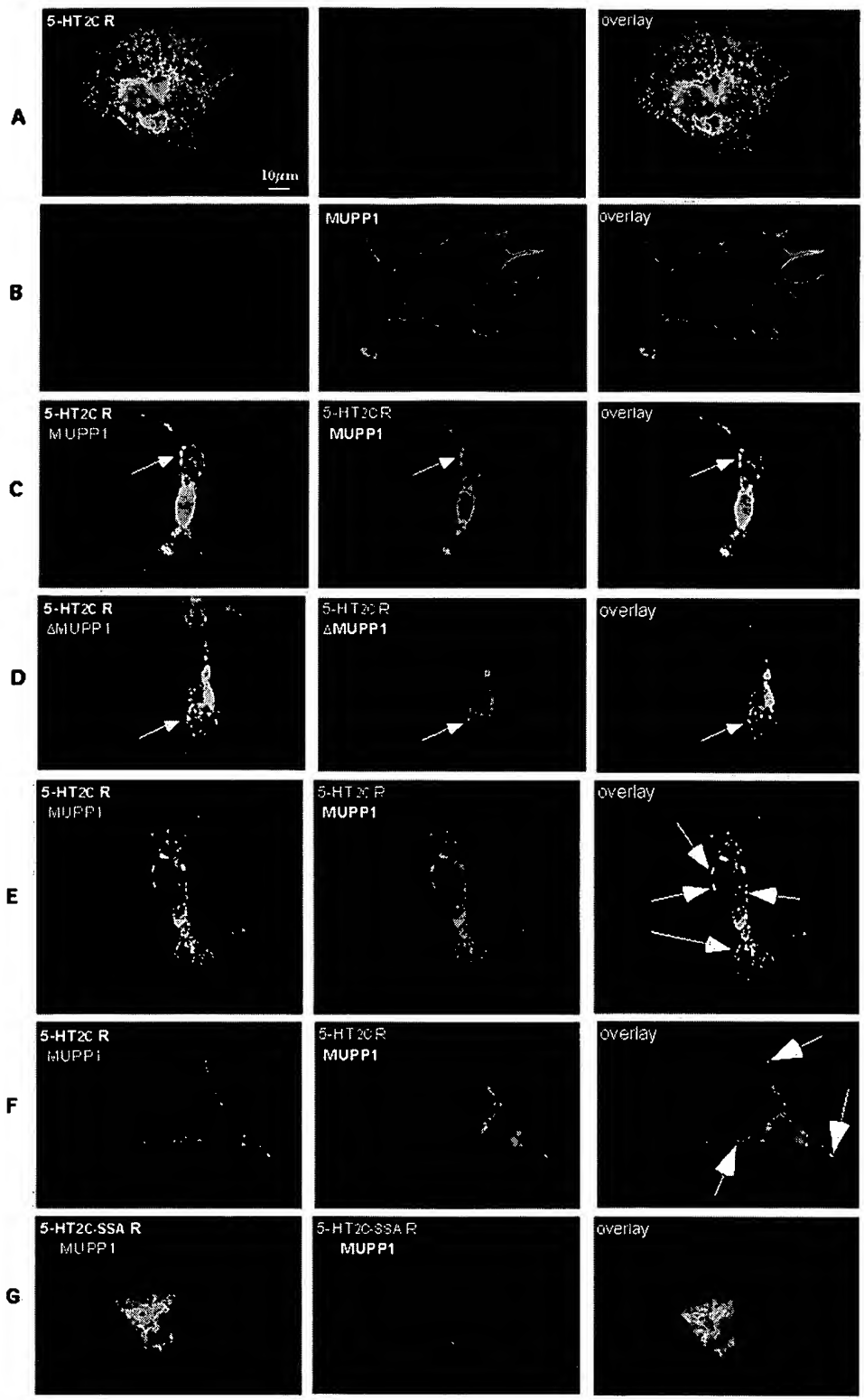


FIG. 4. **MUPP1 induces 5-HT$_{2C}$ receptor clustering in transiently transfected COS-7 cells.** *A*, cells expressing c-Myc-tagged 5-HT$_{2C}$ receptors (*5-HT2C R*) were immunostained using the anti-c-Myc antibody. *B*, cells expressing MUPP1-VSV were immunostained using the anti-MUPP1 antiserum. *C*, in cells expressing c-Myc-tagged 5-HT$_{2C}$ receptors and MUPP1-VSV, both proteins were contained within the same clusters, as indicated by *arrows*. *D*, in cells expressing 5-HT$_{2C}$ receptors and VSV-ΔMUPP1, the same clusters formed as well. *E*, shown is a confocal section of a cell expressing c-Myc-tagged 5-HT$_{2C}$ receptors and MUPP1-VSV. Most clusters were formed intracellularly. *Arrows* indicate clusters at or near the cell-surface membrane. *F*, cells expressing c-Myc-tagged 5-HT$_{2C}$ receptors and MUPP1-VSV were fixed and stained with the anti-N-terminal c-Myc antibody to label surface 5-HT$_{2C}$ receptors (*left panel*). The same cells was then permeabilized and immunostained using the anti-MUPP1 antiserum (*center panel*). *Arrows* indicate clusters at the cell-surface membrane. *G*, cells coexpressing the mutant 5-HT$_{2C\text{-}SSA}$ receptor and MUPP1-VSV failed to form clusters.

thalamic, hypothalamic, and pontine nuclei. In the cerebellum, high levels of transcripts were found in the granular layer. Transcripts were detected in the lateral ventricle, which was due to staining of the epithelial ependymal cells as well as the choroid plexus. These results indicate that *MUPP1* mRNA colocalizes with 5-HT$_{2C}$ receptor expression in all regions of the

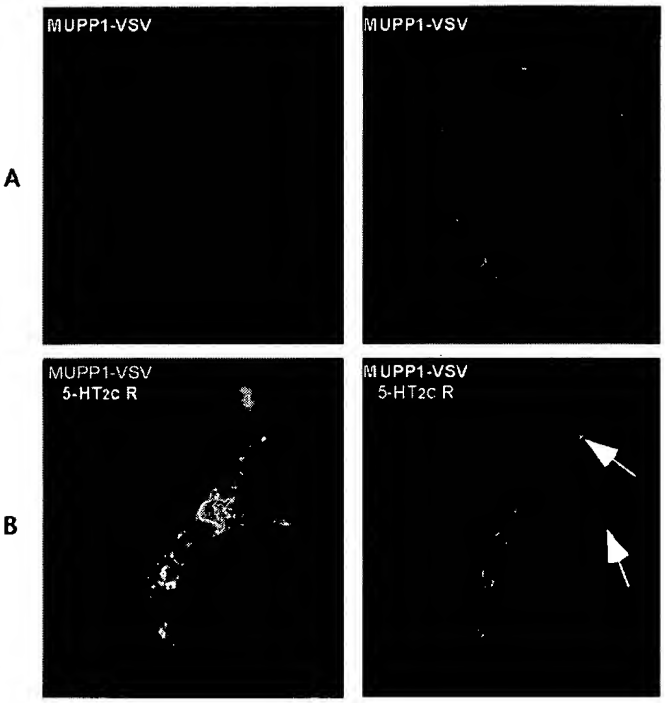


FIG. 5. **5-HT$_{2C}$ receptors unmask the VSV tag of MUPP1-VSV.** *A*, COS-7 cells were transiently transfected with MUPP1-VSV. MUPP1 was detected with the anti-MUPP1 antiserum (*right panel*), but not with the anti-VSV antibody (*left panel*). *B*, in cells expressing 5-HT$_{2C}$ receptors (*5-HT2C R*) and MUPP1-VSV, 5-HT$_{2C}$ receptors were immunostained using the anti-5-HT$_{2C}$ receptor antiserum (*left panel*), and MUPP1 was immunostained using the anti-VSV antibody (*right panel*). 5-HT$_{2C}$ receptors and MUPP1 were contained within the same clusters, indicated by *arrows*.

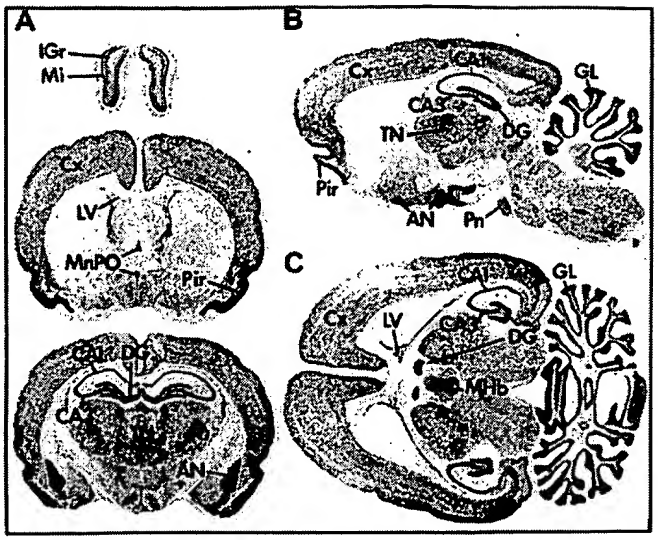


FIG. 6. ***In situ* hybridization analysis of *MUPP1* transcripts in rat brain.** Tissue sections were hybridized to ^{33}P-labeled antisense probes. Shown are representative autoradiograms of coronal sections sliced at bregma 6.7, 0.4, and 3.14 mm, respectively. *A*, sagittal section sliced laterally at 1.9 mm; *B*, a horizontal section at bregma 4.28 nm; *C*, according to the atlas of Ref. 54. Only very weak hybridization signals were observed in the granular layer of the cerebellum with the radiolabeled sense probe (data not shown). *AN*, amygdaloid nuclei; *Cx*, cortex; fields *CA1–CA3* of hippocampus; *DG*, dentate gyrus; *GL*, granular layer; *IGr*, internal granular layer of the olfactory bulb; *LV*, lateral ventricle; *Mi*, mitral cell layer of the olfactory bulb; *MHb*, medial habenular nucleus; *MnPO*, median preoptic nucleus; *Pir*, piriform cortex; *Pn*, pontine nuclei; *TN*, thalamic nuclei.

rat brain (11), including the choroid plexus, where 5-HT$_{2C}$ receptors are highly enriched (38, 39). Immunocytochemistry and confocal microscopy were applied to investigate the colocalization of MUPP1 and 5-HT$_{2C}$ receptor proteins in choroid plexus tissues. Both proteins were expressed on the apical membrane of epithelial choroid plexus cells (Fig. 7, *A* and *B*).

To demonstrate that 5-HT$_{2C}$ receptors and MUPP1 also interact *in vivo*, immunoprecipitation was performed with CHAPS-soluble extracts of rat choroid plexus. Western blot analysis revealed the presence of the MUPP1 protein in the choroid plexus (Fig. 8, *first lane*), which could also be immunoprecipitated with the anti-MUPP1 antiserum directed against the PDZ3 domain (*second lane*). The preimmune serum failed to immunoprecipitate the MUPP1 protein (Fig. 8, *fourth lane*). The anti-5-HT$_{2C}$ receptor antiserum was able to co-immunoprecipitate the MUPP1 protein (Fig. 8, *third lane*), confirming that MUPP1 and the 5-HT$_{2C}$ receptor interact in rat choroid plexus tissues.

Phosphoinositide Hydrolysis Assays—To test possible functional implications of the MUPP1-5-HT$_{2C}$ receptor interaction, serotonin-mediated phosphoinositide hydrolysis was determined in transfected COS-7 cells expressing 5-HT$_{2C}$ receptors in the absence or presence of MUPP1. In the both cases, dose-dependent stimulation of 5-HT$_{2C}$ receptors demonstrated identical EC_{50} values (0.9 ± 0.1 and 0.6 ± 0.1 nM, respectively), with an approximate 4–5-fold increase in basal inositol phosphate accumulation (Fig. 9*A*). The 5-HT$_{2C}$ receptor density as determined by Scatchard analysis of radioligand-saturation binding experiments was on the same order for transfected COS-7 cells in absence or presence of MUPP1 (Fig. 9*B*). These data indicate that the interaction of heterologously expressed 5-HT$_{2C}$ receptors with MUPP1 does not influence receptor expression levels or the second messenger activity of phosphoinositide-mediated phospholipase C activation.

DISCUSSION

The results presented here demonstrate that MUPP1 selectively interacts with the C-terminal SXV motif of the 5-HT$_{2C}$ receptor via PDZ10 *in vitro* and *in vivo*. Using a random tagged fragment library of MUPP1, we showed in yeast that the C terminus of the 5-HT$_{2C}$ receptor selects exclusively PDZ10 for MUPP1 interaction. This interaction requires the complete PDZ10 domain. In agreement with the literature, the 5-HT$_{2C}$ receptor terminating with a serine at position −2 selects the PDZ10 domain, which displays a histidine at the αB1 position (20). His$^{\alpha B1}$ has been shown to coordinate the hydroxyl group of the serine at position −2 of the PDZ-binding motif (40).

Immunoprecipitation of 5-HT$_{2C}$ receptors with CHAPS-soluble extracts of transfected COS-7 cells revealed glycosylated and unglycosylated receptors, which could be co-immunoprecipitated with anti-MUPP1 antibodies. This suggests that MUPP1 interacts with the glycosylated membrane-bound receptor as well as with the unglycosylated intracellular receptor reserve. Immunofluorescence in transfected COS-7 cells revealed a different localization of MUPP1 and 5-HT$_{2C}$ receptors when both were transfected separately. MUPP1 was homogeneously distributed, whereas 5-HT$_{2C}$ receptors localized within membrane-type structures, including intracellular membranes. Coexpression induced the formation of MUPP1 clusters that were strikingly different from the distribution observed with MUPP1 only. This suggests that the clustering within membranous structures was mediated by the presence of 5-HT$_{2C}$ receptors. Concerning the expression at the cell surface, MUPP1 appears to induce clustering of a few 5-HT$_{2C}$ receptors, but the total number of cell-surface receptors remains unchanged. The direct physical interaction of MUPP1 with the PDZ-binding motif of 5-HT$_{2C}$ receptors is manifested by the conformational change triggered within the MUPP1

molecule. The functional importance of the 5-HT$_{2C}$ receptor interaction with MUPP1 remains to be answered since MUPP1 did not influence receptor expression levels or the activation of phospholipase C. However, these experiments were performed in COS-7 cells, which might resemble an artificial environment. Since MUPP1 contains 13 different PDZ domains (20), a maximum of 13 different players have to be taken into account that could potentially link the MUPP1 molecule to specialized submembranous sites, thereby selectively assembling unique signaling complexes. These proteins are unlikely to be completely present in COS-7 cells. Therefore, to investigate the functional relevance of the MUPP1-5-HT$_{2C}$ receptor interaction, tissues that endogenously express both proteins have to be analyzed. Indeed, using confocal microscopy, we demonstrated that MUPP1 and 5-HT$_{2C}$ receptors colocalize exclusively at the apical surface of epithelial cells from choroid plexus tissues. The apical localization is in agreement with an early report suggesting an activation of 5-HT$_{2C}$ receptors by cerebrospinal fluid-borne serotonin (41).

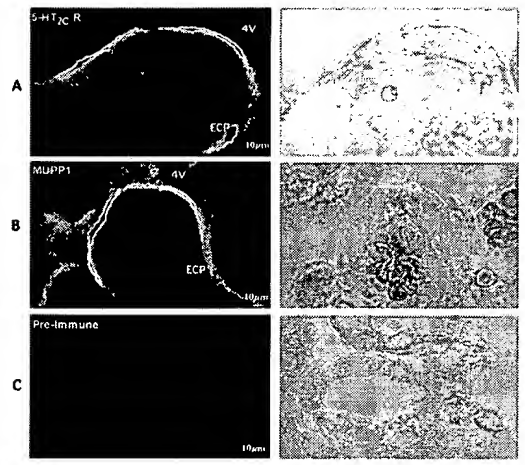
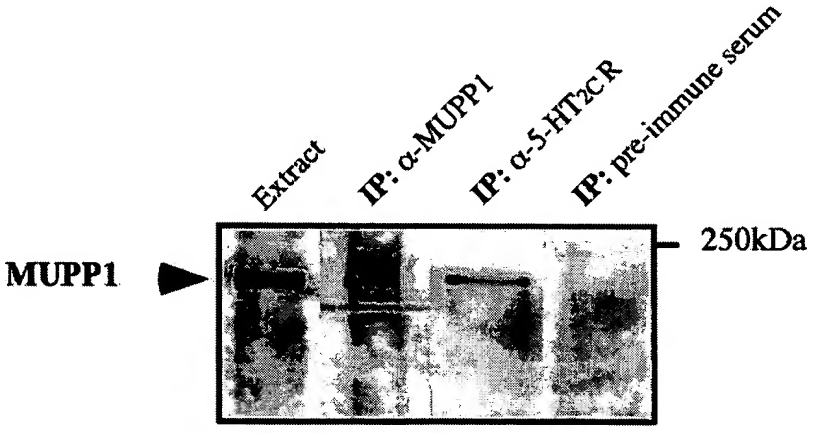


FIG. 7. **Immunohistochemistry of MUPP1 and the 5-HT$_{2C}$ receptor in the choroid plexus.** *A*, immunolabeling of a choroid plexus slice (10 μm) with the anti-5-HT$_{2C}$ receptor (*5-HT$_{2C}$ R*) antibody; *B*, immunolabeling with the anti-MUPP1 antiserum; *C*, immunolabeling with the preimmune antiserum. *4V*, fourth ventricle; *ECP*, epithelial choroid plexus cells.

Human 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors share the C-terminal EX(V/I)SXV sequence with 5-HT$_{2C}$ receptors even though all three 5-HT$_2$ receptors differ greatly in the overall identity of their intracellular C-terminal amino acid sequences. In accordance with the striking conservation of a common PDZ-binding motif at their extreme C terminus, all three receptors bind to MUPP1 PDZ domains *in vitro*. Since we demonstrated the *in vivo* interaction of MUPP1 with the 5-HT$_{2C}$ receptor, interactions with 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors may occur at least in tissues displaying overlapping expression with MUPP1. In contrast to 5-HT$_{2C}$ receptors, which appear to be exclusively expressed in neuronal tissues (42), 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors are also present in peripheral organs. 5-HT$_{2A}$ receptor mRNA has been shown to be present in various human smooth muscle cells (26), and 5-HT$_{2B}$ receptor mRNA in human heart, placenta, liver, kidney, and pancreas (43, 44), tissues that are known to express MUPP1 (20). In rat brain, MUPP1 expression coincides with 5-HT$_{2A}$ transcripts in the cerebral cortex, the

FIG. 8. **Co-immunoprecipitation of MUPP1 and 5-HT$_{2C}$ receptors in CHAPS-soluble extracts of the choroid plexus.** Membrane fractions of rat choroid plexus were solubilized with CHAPS (*first lane*) and immunoprecipitated (*IP*) with the anti-5-HT$_{2C}$ receptor antiserum (*second lane*) or with the anti-MUPP1 antiserum (*third lane*). The immunoprecipitates were analyzed by Western blotting using the anti-MUPP1 antiserum. MUPP1 was not immunoprecipitated by the preimmune serum (*fourth lane*).



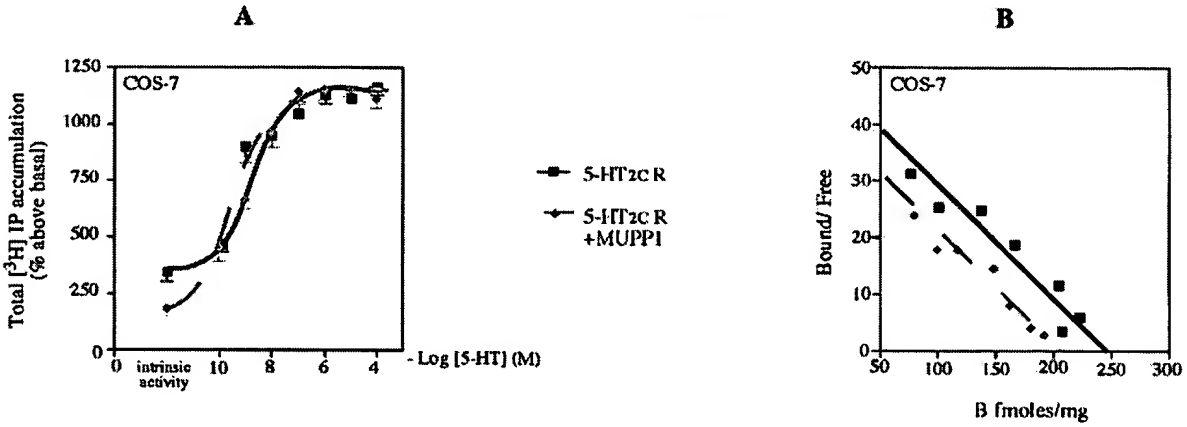


FIG. 9. **Inositol phosphate accumulation and receptor density in the absence and presence of MUPP1.** *A*, stimulation of inositol phosphate (*IP*) accumulation by increasing concentrations of 5-hydroxytryptamine; *B*, Scatchard analysis of saturation experiments of [^{3}H]mesulergine binding to membranes in COS-7 cells transfected with 5-HT$_{2C}$ receptors (*5-HT2C R*) and MUPP1 or with 5-HT$_{2C}$ receptors alone. The results are representative of three experiments performed in triplicate.

olfactory system including the mitral cell layer and the piriform cortex, the CA3 pyramidal cell layer, and the pontine nuclei (45). 5-HT$_{2B}$ receptors are coexpressed with *MUPP1* transcripts in the medial amygdala, dorsal hypothalamus, frontal cortex, and granular layer of the cerebellum (46, 47).

This is the first report of a protein interacting with a serotonin receptor. The presence of a PDZ-binding motif at the C termini of the 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors has been documented (18, 20, 48). To date, there are two reports about possible functions of the C-terminal PDZ-binding motifs of the mouse 5-HT$_{2B}$ (48) and rat 5-HT$_{2C}$ (49) receptors. The serine at position −2 was shown to enhance resensitization of the 5-HT$_{2C}$ receptor responses (49). This critical Ser^{-2} is part of two possible phosphorylation sites contained within the 5-HT$_{2C}$ receptor PDZ-binding motif. The presence of these phosphorylation sites implies that phosphorylation might regulate the capacity of the binding of the PDZ recognition sequence to the MUPP1 PDZ10 domain. Phosphorylation sites for Ser^{-2} within PDZ-binding motifs were also observed for the C terminus of the β_2-adrenergic receptor (50). It has been suggested that G-protein-coupled-receptor kinase-5-mediated phosphorylation may disrupt the interaction with NHERF and thereby regulate the sorting of internalized β_2-adrenergic receptors (4). A similar role for the PDZ-binding motif of the 5-HT$_{2C}$ receptor can be anticipated.

In a murine cell line expressing 5-HT$_{2B}$ receptors, it was demonstrated that stimulation of 5-HT$_{2B}$ receptors triggers an increase in intracellular cGMP through dual activation of constitutive and inducible nitric-oxide synthase. This activity is dependent on the C-terminal PDZ-binding motif (48). 5-HT$_{2C}$ receptors have also been shown to be involved in nitric oxide signaling such as inhibition of the *N*-methyl-D-aspartate/nitric oxide/cGMP pathway in the rat cerebellum (51) and stimulation of cGMP formation in the choroid plexus (52), which in turn inhibits phosphoinositide turnover in the choroid plexus (53). PDZ interactions with MUPP1 could provide the basis for 5-HT$_{2C}$ receptor-mediated cross-talks between second messenger pathways such as phosphoinositide hydrolysis and nitric oxide signaling. By searching for the proteins that interact with the remaining 12 PDZ domains of MUPP1, novel intracellular targets that might contribute to our understanding of 5-HT$_{2C}$ receptor trafficking and/or signaling will be identified.

Acknowledgments—We are grateful to M. Staufenbiel and D. Abramowski (Novartis Pharma AG, Basel, Switzerland) for the 5-HT$_{2C}$ receptor 522 antiserum, M. Lerner-Natoli for assistance with immunohistochemistry experiments, M. Sebben for assistance with the binding experiments, and A. Turner-Madeuf for help in language revision.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 278, No. 24, Issue of June 13, pp. 21576–21583, 2003
Printed in U.S.A.

Agonist-induced Phosphorylation of the Serotonin 5-HT_{2C} Receptor Regulates Its Interaction with Multiple PDZ Protein 1*

Received for publication, October 28, 2002, and in revised form, April 2, 2003
Published, JBC Papers in Press, April 7, 2003, DOI 10.1074/jbc.M210973200

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Multiple PDZ domain protein 1 (MUPP1), a putative scaffolding protein containing 13 PSD-95, Dlg, ZO-1 (PDZ) domains, was identified by a yeast two-hybrid screen as a serotonin$_{2C}$ receptor (5-HT_{2C} R)-interacting protein (Ullmer, C., Schmuck, K., Figge, A., and Lubbert, H. (1998) *FEBS Lett.* 424, 63–68). MUPP1 PDZ domain 10 (PDZ 10) associates with Ser^{458}-Ser-Val at the carboxyl-terminal tail of the 5-HT_{2C} R. Both Ser^{458} and Ser^{459} are phosphorylated upon serotonin stimulation of the receptor (Backstrom, J. R., Price, R. D., Reasoner, D. T., and Sanders-Bush, E. (2000) *J. Biol. Chem.* 275, 23620–23626). To investigate whether phosphorylation of these serines in the receptor regulates MUPP1 interaction, we used several approaches. First, we substituted the serines in the receptor carboxyl tail with aspartates to mimic phosphorylation (S458D, S459D, or S458D/S459D). Pull-down assays demonstrated that Asp mutations at Ser^{458} significantly decreased receptor tail interaction with PDZ 10. Next, serotonin treatment of 5-HT_{2C} R/3T3 cells resulted in a dose-dependent reduction of receptor interaction with PDZ 10. Effects of serotonin on receptor-PDZ 10 binding could be blocked by pretreatment with a receptor antagonist. Alkaline phosphatase treatment reverses the effect of serotonin, indicating that agonist-induced phosphorylation at Ser^{458} resulted in a loss of MUPP1 association and also revealed a significant amount of basal phosphorylation of the receptor. We conclude that 5-HT_{2C} R interaction with MUPP1 is dynamically regulated by phosphorylation at Ser^{458}.

A growing number of proteins containing PDZ[1] domains have been shown to play important roles in the organization and/or regulation of signaling events in cells. PDZ domains (or GLGF repeats) were named after three proteins identified over a decade ago: postsynaptic density-95, *Drosophila* Discs large, and zonula occludens-1 (3–5). These three proteins belong to the membrane-associated guanylate kinase (MAGUK) family of proteins. Most MAGUK proteins contain three PDZ domains, an Src homology 2 domain, and a guanylate kinase-like domain, each having different cellular roles. PDZ domains range from 80 to 100 amino acids in length and typically bind to the carboxyl-terminal sequence of target proteins including receptors, channels, and various signaling molecules to regulate subcellular localization, trafficking, recycling, and/or signaling (6–10).

MUPP1, a protein containing 13 putative PDZ domains, was isolated in a yeast two-hybrid screening for proteins that bound to the carboxyl-terminal tail of the 5-HT_{2C} R (1). MUPP1 is expressed in many tissues, whereas the 5-HT_{2C} R is a brain-specific protein (1, 11). The 5-HT_{2C} R has classically been thought to couple to G_q activation; however, additional G protein families have been implicated, leading to the activation of different downstream signaling pathways including phospholipase A_2, C, or D, and various cation channels (12–17). Since PDZ-containing proteins can scaffold many signaling molecules together into a signal transduction complex, the interaction between MUPP1 and the 5-HT_{2C} R was further investigated. The 5-HT_{2C} R contains a PDZ binding motif, Ser^{458}-Ser-Val, at its extreme carboxyl terminus, which is critical for interaction with PDZ 10 of MUPP1 (18). In an alternate approach to the yeast two-hybrid system, we independently show that PDZ 10 of MUPP1 is the primary site of interaction for the 5-HT_{2C} R.

Serotonin stimulation has previously been shown to promote phosphorylation of the two serine residues of the 5-HT_{2C} R PDZ binding motif, Ser^{458} and Ser^{459} (2). We therefore hypothesize that phosphorylation of the carboxyl-terminal serines of the 5-HT_{2C} R regulates receptor interaction with MUPP1. To test this hypothesis, we investigated whether a modification of Ser^{458} and/or Ser^{459} of the 5-HT_{2C} R carboxyl-terminal tail would alter PDZ 10 interaction. Ser^{458} and/or Ser^{459} of the receptor tail were mutated to aspartate to mimic phosphorylation (*i.e.* introduction of a negative charge). Next, cells expressing 5-HT_{2C} Rs were treated with agonist or antagonist to assess the interaction of the 5-HT_{2C} R with MUPP1. The results of these experiments support our hypothesis that phosphorylation is a key regulator of 5-HT_{2C} R interaction with MUPP1. Furthermore, the results indicated that a significant amount of basal phosphorylation of the receptor may also play a yet undetermined role in regulating PDZ-protein interactions.

MATERIALS AND METHODS

Antibodies

Polyclonal anti-peptide antibodies against amino acids 419–435 (amino acids RHTNERVARKANDPEPG) of the rat 5-HT_{2C} R were generated as described previously (19). Anti-glutathione *S*-transferase (GST) antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

* This work was supported by National Institutes of Health (NIH) Grants EY09743 (to B.-H. S.) and MH34007 (to E. S.-B.), a grant from the National Alliance for Research on Schizophrenia and Depression (to J. R. B.), and NIGMS, NIH Minority Access to Research Careers predoctoral fellowship F31 GM20275 (to L. L. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PDZ, postsynaptic density-95, Discs large, zonula occludens-1; MUPP1, multiple PDZ domain protein 1; 5-HT_{2C} R, serotonin$_{2C}$ receptor; MAGUK, membrane-associated guanylate kinase; GST, glutathione *S*-transferase; BOL, 2-bromo-lysergic acid diethylamide; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate; WT, wild type.

DNA Constructs

Overlapping regions of MUPP1 containing two or three PDZ domains (Fig. 1), or one PDZ domain (PDZ 9, 10, or 11) were generated by reverse transcription-PCR, sequenced, and subcloned into pGEX-4T1 (Amersham Biosciences) for expression of GST fusion proteins. MUPP1 PDZ 9, 10, or 11 were also subcloned into pGEMEX-1 (Promega), a T7 gene 10 fusion protein vector. MUPP1 PDZ 9–11 was also subcloned into pcDNA3 (Invitrogen). The 5-HT$_{2C}$ R carboxyl-terminal tail (last 60 amino acids) with or without the PDZ binding motif (Ser^{458}-Ser-Val) and a truncation mutant at residue 445 were subcloned into pGEMEX-1. The 5-HT$_{2C}$ R carboxyl-terminal tail with the PDZ binding motif was also subcloned into pGEX-4T1. The 5-HT$_{2C}$ R carboxyl tail Ser^{458}-Ser-Val (WT) was modified to S458A, S458D, S459D, or S458D/S459D by PCR site-directed mutagenesis and subcloned into pGEMEX-1.

GST Fusion Protein Overexpression

Escherichia coli was transformed with pGEX-4T1 constructs, induced to overexpress fusion proteins with isopropyl β-D-thiogalactoside, and analyzed. Bacterial lysates were obtained by first adding cold lysis buffer (50 mM Tris pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 mM benzamide, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride) to resuspend the pellets. Resuspended pellets were sonicated for 20 s on ice and centrifuged at 15,000 rpm for 30 min at 4 °C. Proteins were resolved on SDS-PAGE to confirm overexpressed GST fusion protein by Coomassie Blue staining and Western blotting using GST antibodies.

[^{35}S] in Vitro Translation

pGEMEX-1 constructs were used for coupled transcription and translation using the TnT® *in vitro* translation system (Promega) in the presence of [^{35}S]methionine (PerkinElmer Life Sciences) according to the supplier's protocol to generate ^{35}S-labeled proteins.

Protein Overlay Assays

Ten micrograms of GST fusion proteins or GST were size-fractionated on SDS-PAGE and transferred onto nitrocellulose membrane. Nitrocellulose membranes were blocked with freshly prepared 1% BSA/phosphate-buffered saline for 1 h at room temperature. Solution was then replaced with ^{35}S-labeled fusion proteins in 1% BSA/phosphate-buffered saline buffer and incubated with nitrocellulose membranes for 16 h at 4 °C. Nitrocellulose membranes were rinsed three times for 20 min at room temperature in 1% BSA/phosphate-buffered saline containing 0.2% Triton X-100. Nitrocellulose membranes were air-dried and exposed to x-ray film or a PhosphorImager screen (Amersham Biosciences) to visualize radiolabeled proteins. Western blot analysis using GST antibodies was used to document similar GST protein levels.

Cell Culture

NIH-3T3 cells stably transfected with the 5-HT$_{2C}$ R (5-HT$_{2C}$ R/3T3) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) until confluent (20). Cells were washed four times with Hanks' buffered saline solution (with Ca^{2+}/Mg^{2+}) and then serum starved in serum-free Dulbecco's modified Eagle's medium for 16 h. Cells were treated without or with antagonist (1 μM 2-bromolysergic acid diethylamide (BOL)) for 15 min at 37 °C prior to serotonin addition for 30 min at 37 °C. Medium was then removed, 1 ml of Tris buffer (50 mM Tris, pH 7.6, 0.5 mM EDTA, pH 8.0, 5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride) was added, and cells were scraped from plates and placed in an Eppendorf tube on ice. Membrane extracts were obtained using 300 μl of Tris buffer containing 1% Triton X-100. Membrane protein concentrations were determined by BCA protein assay (Pierce). Equal amounts of protein were added to the affinity columns. Western blot analysis using GST antibodies was used to determine that similar levels of fusion protein were pulled down.

Pull-down Assays

Twenty microliters of glutathione-Sepharose beads (Amersham Biosciences) were washed three times with PD buffer (20 mM HEPES, pH 7.6, 100 mM KCl, 10% glycerol, 0.5 mM EDTA, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1% Nonidet P-40). Ten micrograms of GST fusion proteins or GST were incubated with the washed glutathione beads for 1 h at 4 °C. Five microliters of ^{35}S-labeled fusion proteins or 50 μg of membrane extracts were added to the GST-glutathione beads and incubated for 2–3 h or overnight, respectively, at 4 °C. After incubation, GST-glutathione beads were washed six times (for assays with ^{35}S-labeled fusion proteins) or three times (for assays with membrane extracts) with PD buffer. For pull-downs from 5-HT$_{2C}$ R/3T3 cell lysates, precipitated protein, containing the 5-HT$_{2C}$ R, was treated with peptide:*N*-glycanase F before SDS-PAGE (see below). Loading dye (6% SDS, 1% β-mercaptoethanol, 20 mM Tris, pH 6.8, 10% glycerol plus a little bromphenol blue) was added to elute proteins. Eluates were separated on SDS-PAGE and transferred onto nitrocellulose membranes. Autoradiography or PhosphorImager screen was used to visualize radiolabeled proteins. Western blot analysis using GST and 5-HT$_{2C}$ R antibodies were used to document GST fusion proteins and 5-HT$_{2C}$ R, respectively.

Agonist Washout

After serum starvation, cells were treated with 100 nM serotonin for 30 min at 37 °C. After treatment, medium was aspirated to remove the serotonin, and cells were washed four times with Hanks' buffered saline solution. Serum-free Dulbecco's modified Eagle's medium was then added, and cells were incubated for 10 or 30 min. Cells were then lysed, and a pull-down assay was performed as mentioned above.

Band Shift Phosphorylation Assay

This assay was performed as previously described by Backstrom *et al.* (2). Briefly, cells grown to confluence were serum-starved and treated with increasing amounts of serotonin for 15 min at 37 °C. This incubation time was previously shown to be the minimal amount of time that would result in maximal receptor phosphorylation. Cells were then lysed, and membrane extracts containing receptors were prepared. Western blot analysis using 5-HT$_{2C}$ R antibodies were used to document changes in 40- and 41-kDa bands, representative of unphosphorylated and phosphorylated 5-HT$_{2C}$ R, respectively.

Deglycosylation

Following pull-downs from membrane extracts, beads were pelleted and washed once with PD buffer containing 0.1% SDS. Fifteen microliters of PD buffer containing 1% SDS was added to the beads, and the mixture was incubated for 15 min at 37 °C. Then 58 μl of PD buffer was added, and after mixing, 15 μl of PD buffer containing 10% Triton X-100 was added. Finally, 2 μl of peptide:*N*-glycanase F (Glyko or New England Biolabs) was added, and samples were incubated for 2 h at 37 °C. After deglycosylation, 15 μl of 4× loading dye were added, and samples were incubated at room temperature for 20 min prior to SDS-PAGE.

Dephosphorylation

Membrane extracts (50 μg) of untreated or serotonin-treated cells were incubated in PD buffer plus 50 units of calf intestinal (alkaline) phosphatase (New England Biolabs) for 2 h at room temperature. After incubation, pull-down assays were carried out as described above.

Western Blot Analysis

Alkaline Phosphatase Detection—Nitrocellulose membranes were blocked in 1% BSA/Tris blot buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 0.05% NaN_3) for 1 h at room temperature. Membranes were then incubated with GST (1:1000 dilution) or 5-HT$_{2C}$ R (3–5 μg/ml) antibodies in 1% BSA/Tris blot buffer for 2 h to overnight at 4 °C. Membranes were washed three times with Tris blot buffer alone for 10 min. Alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies (1:1000 dilution; Jackson Immunolaboratories) were incubated with membranes for 2 h at room temperature. Membranes were washed three times with Tris blot buffer and once with Tris (150 mM pH 9.4) and then developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium.

Chemilluminescence Detection—Nitrocellulose membranes were blocked in 8% milk/Tween 20 Tris buffer solution (25 mM Tris, pH 7.4, 137 mM NaCl, 0.27 mM KCl, 0.05% Tween 20) overnight at 4 °C. Membranes were then incubated with GST (1:4000 dilution) or 5-HT$_{2C}$ R (0.5 μg/ml) antibodies in 2% milk/Tween 20 Tris buffer solution overnight at 4 °C. Membranes were washed four times for 5 min with Tween 20 Tris buffer solution. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:20,000 dilution; Jackson Immunolaboratories) were incubated with membranes for 45 min at room temperature. Membranes were washed four times for 15 min with Tween 20 Tris buffer solution and developed with the Pierce Supersignal West Dura® kit according to the supplier's protocol. Horseradish peroxidase signal was analyzed by Bio-Rad Flouro-S, and densitometric analysis was performed by QuantityOne (Bio-Rad) software.

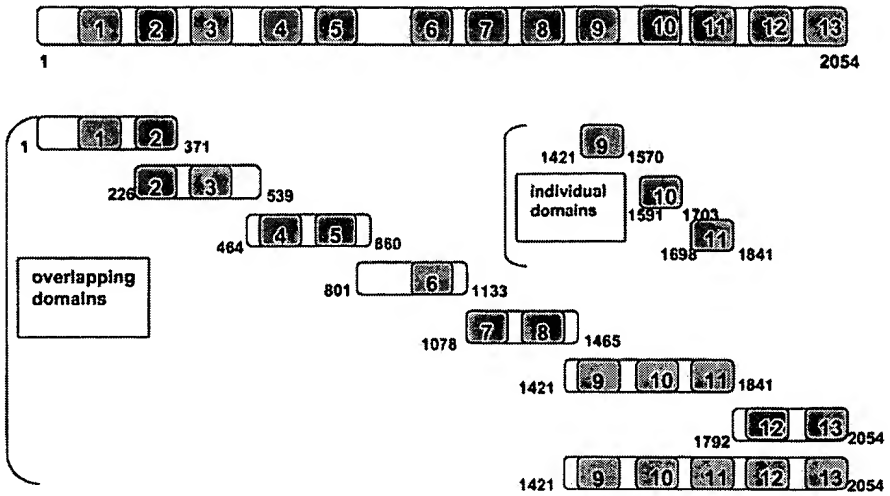


FIG. 1. **Schematic diagram of MUPP1 PDZ domains.** MUPP1 has 2054 residues. Each *numbered block* (*grey*) represents the 13 individual PDZ domains of MUPP1. Pairs or individual PDZ domains were generated as GST or T7 gene 10 fusion proteins to test for 5-HT$_{2C}$ R interaction. *Numbers under* each *block* denote amino acid range.

Statistical Analysis

All bar graph data was analyzed with Graphpad Prism one-way analysis of variance with Tukey's post-test; $p < 0.05$ is significant, unless otherwise noted in a figure legend. GST alone was the background control for all GST fusion protein experiments, and graph data presented are background-subtracted. Data represent the means ± S.D. from several independent experiments.

RESULTS

The 5-HT$_{2C}$ Receptor Selectively Interacts with MUPP1 PDZ 10—The carboxyl region of MUPP1 containing the last four PDZ domains (PDZ 10 to PDZ 13) was originally identified by yeast two-hybrid screening for 5-HT$_{2C}$ R-interacting proteins (1). However, it was unclear which PDZ domain interacted with the 5-HT$_{2C}$ R. Therefore, we set out to identify which domain(s) of MUPP1 interacts with the 5-HT$_{2C}$ R. Overlapping PDZ domain regions of MUPP1 were generated as GST fusion proteins (Fig. 1). Purified GST-MUPP1 PDZ domains were used to pull-down *in vitro* translated ^{35}S-labeled 5-HT$_{2C}$ R carboxyl terminus fusion protein, which consists of the last 60 amino acids harboring a PDZ binding motif, Ser^{458}-Ser-Val. Fig. 2*A* illustrates that significantly more receptor tail interacted with PDZ 9–11 and PDZ 9–13. A weak interaction of the receptor tail over GST alone was observed with PDZ 12 and 13, suggesting that PDZ 9–11 is the primary 5-HT$_{2C}$ R interacting region. In protein overlay assays, GST-MUPP1 PDZ domain fusion proteins were blotted and probed with ^{35}S-labeled 5-HT$_{2C}$ R carboxyl terminus, and the 5-HT$_{2C}$ R tail specifically interacted with PDZ 9–11 and PDZ 9–13; no other PDZ domains displayed a significant interaction with the receptor tail (results not shown). Thus, both protein overlay and pull-down assays consistently indicate that PDZ 9–11 is responsible for interacting with the 5-HT$_{2C}$ R tail.

To further determine the specific site of interaction, GST fusion proteins of the individual PDZ domains, 9, 10, and 11 were made (Fig. 1). Unfortunately, GST-PDZ 10 was unstable when overexpressed in bacteria. Therefore, the ability to pull down the ^{35}S-labeled 5-HT$_{2C}$ R tail by GST-PDZ 9 or 11 was compared with GST-PDZ 9–11. GST fusion proteins of PDZ 9 or 11 alone were not able to bind to the receptor tail as compared with GST-PDZ 9–11, which contains PDZ 10 (data not shown). In a complementary pull-down experiment, the individual MUPP1 PDZ domains 9, 10, and 11 were *in vitro* translated with [^{35}S]methionine and pulled down by the 5-HT$_{2C}$ R carboxyl-terminal tail expressed as a GST fusion protein (Fig. 2*B*). The carboxyl tail of the receptor specifically interacted

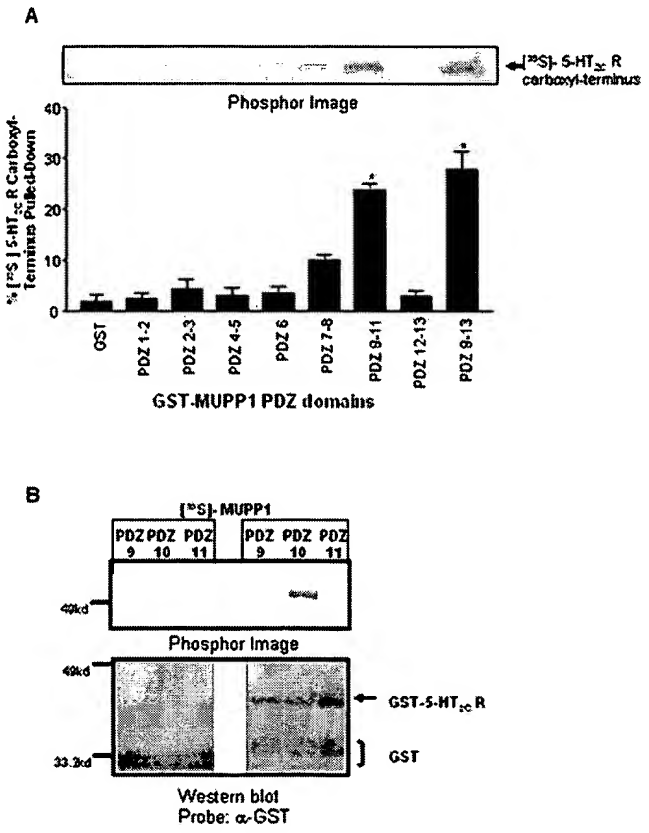


FIG. 2. **MUPP1 PDZ 10 interacts with the carboxyl terminus of the 5-HT$_{2C}$ R.** Pull-down assays were used to determine which MUPP1 PDZ domain(s) interacted with the receptor. *A*, GST-MUPP1 PDZ domains bound to glutathione beads were incubated with [^{35}S]5-HT$_{2C}$ R carboxyl tail. Radioactive fusion receptor tail that bound to specific PDZ domains was resolved by SDS-PAGE, transferred onto nitrocellulose, and analyzed by autoradiography or PhosphorImager. Both PDZ 9–11 and PDZ 9–13 bound significantly more radiolabeled receptor than other PDZ domains. Similar amounts of fusion proteins were used as demonstrated by Western blotting (not shown). The *bar graph* represents $n = 6$; *, $p < 0.001$ relative to GST. The *panel above* the *graph* is a representative pull-down result. *B*, the last 60 amino acids of the 5-HT$_{2C}$ R were used to pull down [^{35}S]MUPP1 PDZ domain 9, 10, or 11. Radioactive PDZ domain pulled down was analyzed by autoradiography or PhosphorImager. *Top panel*, PDZ 10 was specifically pulled down by the 5-HT$_{2C}$ R carboxyl tail fusion but not by GST alone. Equal amounts of GST fusion or GST proteins were shown by Western blotting ($n = 2$; *bottom panel*).

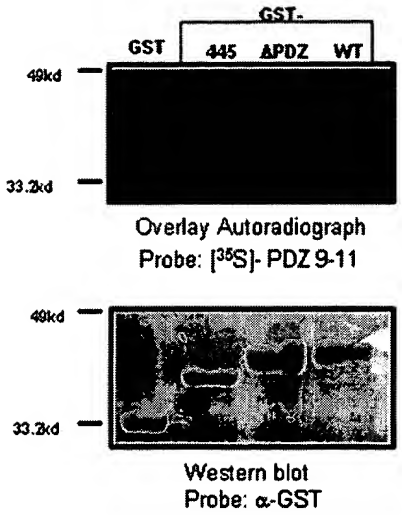
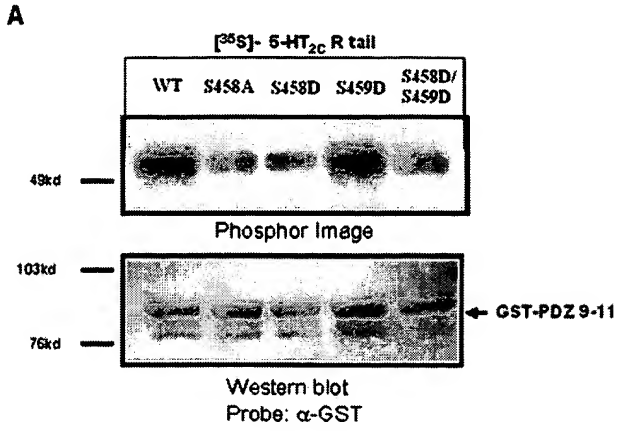


FIG. 3. **The terminal residues of the 5-HT$_{2C}$ R are necessary for PDZ 10 interaction.** Protein overlay assay was employed to assess what region of the carboxyl-terminal tail of the 5-HT$_{2C}$ R is needed for binding to MUPP1. GST fusion proteins of the 5-HT$_{2C}$ R carboxyl tails with the PDZ binding motif (*WT*), without the PDZ motif (*Δ PDZ*), and a truncated mutant at amino acid 445 (*445*) were separated by SDS-PAGE and transferred onto nitrocellulose blots. [^{35}S]MUPP1 PDZ 9–11 was incubated with nitrocellulose blots, and then blots were exposed to film after the removal of nonspecific binding. MUPP1 PDZ 9–11 positively interacted with WT 5-HT$_{2C}$ R carboxyl tail, whereas Δ PDZ and 445 did not exhibit any detectable binding (*top panel*). A nonspecific band was observed that is also present in the GST alone lane. Western blotting was used to show the presence of fusion protein (n = 3) (*bottom panel*).



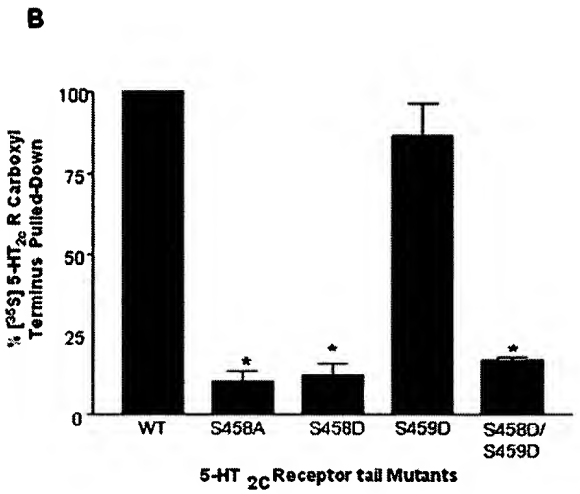


FIG. 4. **Aspartate mutation in the PDZ binding motif of the 5-HT$_{2C}$ R reduces MUPP1 interaction.** Pull-down assays were used to determine whether mutations of serines in the 5-HT$_{2C}$ R carboxyl tail would affect MUPP1 PDZ 10 interaction. *A*, PDZ 9–11 was used to pull down [^{35}S]5-HT$_{2C}$ R carboxyl tails: WT (Ser^{458}-Ser-Val), S458A, S458D, S459D, or S458D/S459D. Mutations at Ser^{459} in the receptor maintained interaction comparable with wild-type interaction with PDZ 9–11, whereas mutations at Ser^{458} caused a significant loss of interaction. *Top panel*, a representative pull-down result. *Bottom panel*, Western blotting of the same filter to detect the amount of PDZ 9–11. *B*, a *bar graph* shows relative interaction compared with WT receptor tail. *, $p < 0.001$. Data were normalized to WT receptor tail, n = 6.

with PDZ 10 and not PDZ domain 9 or 11, further supporting PDZ 10 as the interacting region for the receptor tail.

Next, we questioned whether regions upstream of the extreme carboxyl terminus of the 5-HT$_{2C}$ R are able to confer binding to PDZ 10. To address this question, we generated GST fusion proteins of the 5-HT$_{2C}$ R carboxyl-terminal tail missing only the last three residues (Δ PDZ) and the 5-HT$_{2C}$ R carboxyl-terminal tail ending at residue 445 (*i.e.* missing the last 15 amino acids). In an overlay assay, [^{35}S]PDZ 9–11 was incubated with the different GST-5-HT$_{2C}$ R carboxyl-terminal tail fusion proteins. As illustrated in Fig. 3, PDZ 9–11 binds to WT but not the 5-HT$_{2C}$ R carboxyl-terminal truncation mutants.

Mutation of Ser^{458} in the 5-HT$_{2C}$ Receptor Reveals Altered PDZ 10 Interaction—Studies previously demonstrated that Ser^{458} and Ser^{459} at the extreme carboxyl tail of the 5-HT$_{2C}$ R, the same region of the receptor necessary for PDZ 10 binding, are phosphorylated upon ligand activation (2). A function for Ser^{459} phosphorylation in receptor resensitization was proposed; however, the role for Ser^{458} phosphorylation is unknown. Based upon crystal structures of PDZ domains (21–25) and data compiled on PDZ binding motifs (26), Ser^{458} of the 5-HT$_{2C}$ R is predicted to be a critical residue for interacting with PDZ 10. We therefore hypothesized that agonist-mediated phosphorylation of Ser^{458} disrupts the interaction of MUPP1 PDZ domain 10 and its target, the 5-HT$_{2C}$ R. To test this hypothesis, the serine residues in the receptor tail were replaced with aspartic acid to mimic phosphorylation. The last two serine residues of the 5-HT$_{2C}$ R carboxyl tail (Ser^{458}-Ser^{459}) were modified by PCR site-directed mutagenesis to contain S458A, S458D, S459D, or S458D/S459D substitutions. Wild-type and mutated 5-HT$_{2C}$ R tails were labeled with [^{35}S]methionine and incubated with GST-PDZ 9–11. 5-HT$_{2C}$ R tail mutants containing S458A, S458D, and S458D/S459D substitutions displayed a marked loss of interaction to PDZ 9–11 (Fig. 4*A*). The S459D mutation, however, retained an ability to interact similar to wild-type interaction (Fig. 4*B*). These results indicate that Ser^{458} is an important residue in determining the interaction with PDZ 10.

Serotonin Treatment Decreases the Ability of the 5-HT$_{2C}$ Receptor to Interact with PDZ 10—Results from the 5-HT$_{2C}$ R tail mutants raise the possibility of a dynamic regulation of the interaction between the 5-HT$_{2C}$ R and MUPP1. Thus, we investigated whether agonist stimulation of the 5-HT$_{2C}$ R stably expressed in NIH-3T3 cells would also result in a loss of MUPP1 interaction. To determine whether serotonin stimulation had any effect on MUPP1-receptor interaction, cells were incubated with increasing amounts of serotonin, which have been shown to promote receptor phosphorylation (2). The ability of the 5-HT$_{2C}$ R to bind to PDZ 10 was assessed by pull-down assays. Fig. 5*A* shows that cells treated with serotonin led to a dose-dependent decrease in receptor interaction with MUPP1. A 50% reduction in receptor binding to PDZ 10 was observed with a concentration 100 nM serotonin. Moreover,

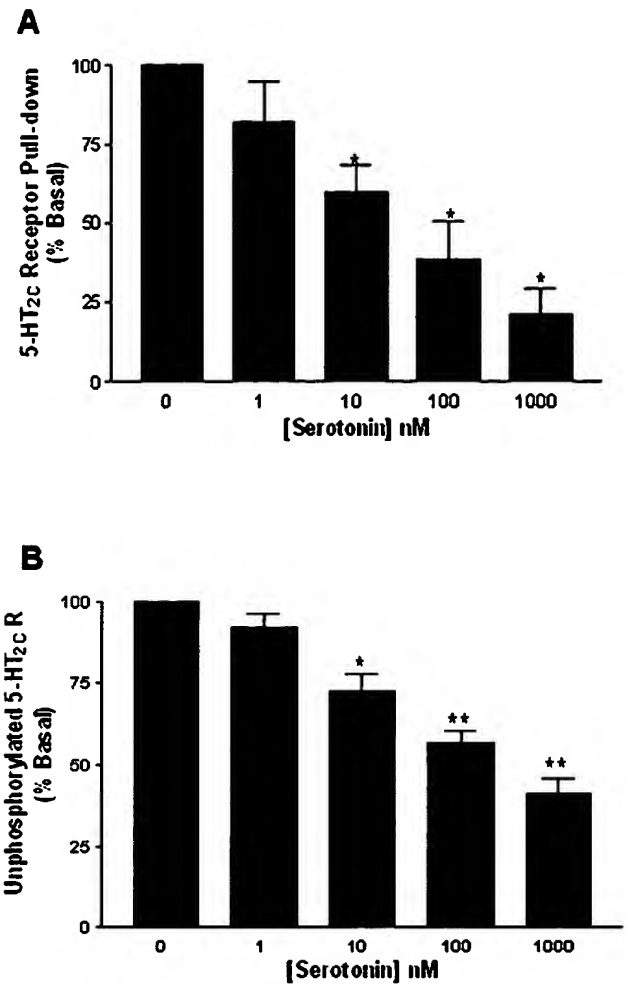


FIG. 5. **Serotonin treatment decreases 5-HT$_{2C}$ R interaction with MUPP1 in a dose-dependent manner.** Serotonin treatment demonstrates a correlation between receptor interaction with MUPP1 and receptor phosphorylation state. *A*, membrane extracts from 5-HT$_{2C}$ R/3T3 cells treated with various concentrations of serotonin (1–1000 nM) were incubated with MUPP1 PDZ 9–11. 5-HT$_{2C}$ R bound to PDZ 9–11 was deglycosylated and analyzed by SDS-PAGE followed by Western blot analysis to determine the amount of 5-HT$_{2C}$ R pulled down. Amount of receptor recovered was compared and normalized to receptor from untreated cells. The *bar graph* represents $n = 4$; *, $p < 0.001$. *B*, band shift phosphorylation assay revealed a concentration-dependent decrease in levels of unphosphorylated receptor. A 40-kDa band was previously determined to be an unphosphorylated form of the receptor, and the 41-kDa band was the phosphorylated form. Changes in mobility of the receptor from the 40- to 41-kDa band were compared and quantitated after increasing amounts of serotonin treatment. Data were normalized to the 40-kDa (unphosphorylated) receptor band from untreated cells. The *bar graph* represents $n = 3$; *, $p < 0.01$; **, $p < 0.001$.

increasing serotonin concentrations caused a dose-dependent increase in phosphorylated receptor with a concurrent decrease in the amount of unphosphorylated receptor as determined by band shift phosphorylation assays (Fig. 5*B*).

To determine whether the loss of PDZ 10 interaction with the receptor was a consequence of agonist binding with 5-HT$_{2C}$ Rs, cells were preincubated in the absence or presence 1 μM of BOL, a 5-HT$_{2C}$ R antagonist, for 15 min prior to the addition of serotonin. BOL antagonized a subsequent serotonin-mediated decrease in receptor pull-down (Fig. 6), thereby demonstrating that the loss of PDZ 10 interaction is a direct consequence of receptor activation. BOL alone had no effect.

Alkaline Phosphatase Treatment of the 5-HT$_{2C}$ Receptor Increases PDZ 10 Interaction and Reveals 5-HT$_{2C}$ Receptor Basal Phosphorylation—The reduction of 5-HT$_{2C}$ R binding to

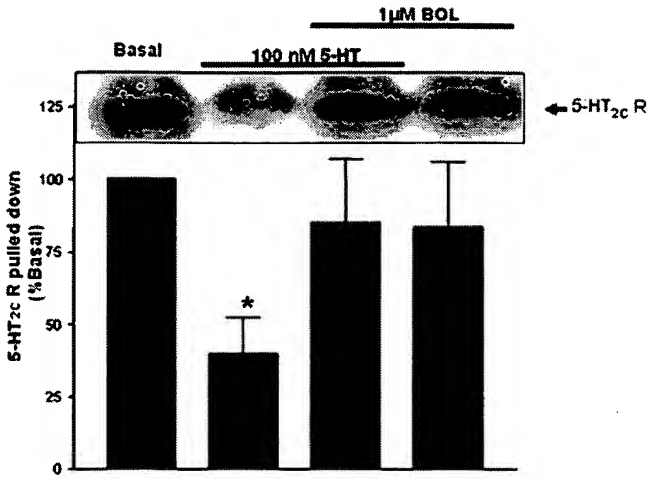


FIG. 6. **Serotonin effects can be modulated by 5-HT$_{2C}$ R antagonist treatment.** Pull-down assays from 5-HT$_{2C}$ R/3T3 cells show that receptor interaction with MUPP1 can be modulated by BOL. Membrane lysates from untreated cells or cells treated with 100 nM serotonin in the absence or presence of antagonist (1 μM BOL) were incubated with PDZ 9–11. BOL blocks the effects of 100 nM serotonin on 5-HT$_{2C}$ R binding to PDZ 9–11, whereas BOL alone has no effects. Chemiluminescence using a 5-HT$_{2C}$ R-specific antibody was used to compare the amount of receptor protein pulled down. The amount of receptor pulled down was normalized to untreated receptor lysate (control); $n = 6$; *, $p < 0.01$. The *panel above* the *bar graph* is a representative experiment.

MUPP1 may be the direct result of receptor phosphorylation. We therefore investigated whether treatment of lysate containing receptor with alkaline phosphatase would restore MUPP1 interaction. Cells were treated with agonist, and cell lysates were incubated with alkaline phosphatase prior to pull-down assays. As shown in Fig. 7*A*, alkaline phosphatase treatment resulted in more receptor pull-down in serotonin-stimulated cells. In the absence of serotonin, alkaline phosphatase treatment doubled the amount of receptor binding to PDZ 10 compared with untreated cells. These findings directly support a role for agonist-induced phosphorylation in disrupting 5-HT$_{2C}$ R binding to MUPP1 as well as uncover a potential function for previously reported basal phosphorylation of the receptor.

Phosphorylation of the receptor is reversible; therefore, we investigated the activity of endogenous phosphatases against the receptor by washout experiments. Cells were treated with agonist and then washed thoroughly and incubated in serum-free medium for 10 or 30 min before lysis and pull-down assay. Fig. 7*B* demonstrates a time-dependent increase in 5-HT$_{2C}$ R binding to MUPP1 (Fig. 7*B*). These results are consistent with previously published data indicating a time-dependent dephosphorylation of the receptor (2).

DISCUSSION

5-HT$_{2C}$ Rs are implicated in physiological processes such as cerebrospinal fluid production as well as illnesses and disorders including anxiety, migraines, and eating and sleeping disorders (27, 28). Furthermore, the 5-HT$_{2C}$ R is a target for hallucinogenic drugs such as lysergic acid diethylamide (29, 30). A thorough understanding of intracellular signaling by the 5-HT$_{2C}$ R, including its interaction with PDZ domain containing proteins, may give insight into the cellular mechanisms that underlie these diverse physiological processes. PDZ domain-containing proteins are involved in the localization of potassium channels and glutamate receptors in the synapse as well as localization of numerous other receptors and proteins (31–36). PDZ domains typically bind to the carboxyl termini of target proteins that contain a PDZ binding motif. Interestingly, some PDZ binding motifs have been shown to be phosphorylated (37–47), including the 5-HT$_{2C}$ R (2).

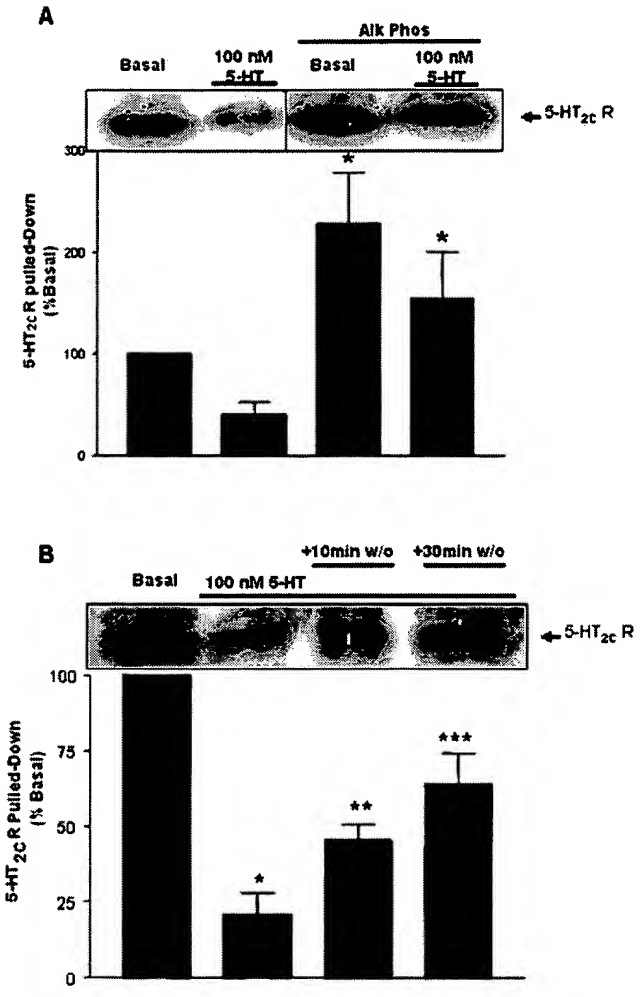


FIG. 7. **Phosphatase and agonist washout restore 5-HT$_{2C}$ receptor interaction with PDZ 10.** *A*, membrane lysate from cells treated without or with agonist (100 nM serotonin) was incubated with 1000 units/mg of protein of alkaline phosphatase and then incubated with PDZ 9–11. Alkaline phosphatase treatment resulted in an increase in the amount of receptor pulled down from extracts prepared from untreated and serotonin-treated cells. *B*, the effect of agonist washout (10 or 30 min) following 100 nM serotonin treatment was investigated. Membrane lysates from these cells were incubated with PDZ 9–11. A time-dependent increase of the MUPP1 interaction following serotonin washout was observed. For both graphs, the amount of receptor pulled down was normalized to untreated receptor lysate (control); $n = 6$ or 3, respectively; *, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.05$. The *panels above* the *bar graphs* are representative experiments.

In the current paper, 5-HT$_{2C}$ R interaction with PDZ 10 of MUPP1 was independently confirmed by using pull-down and protein overlay assays. These results are consistent with the report by Becamel *et al.* (18) that demonstrated PDZ 10 interaction with the 5-HT$_{2C}$ R in yeast two-hybrid assays. Becamel *et al.* (18) also demonstrated that mutation of the critical residues of the PDZ motif on the receptor, Ser^{458} or Val^{460} to alanine, abolished interaction with PDZ 10. Similarly, our results also document that the carboxyl-terminal residues of the 5-HT$_{2C}$ R are necessary for MUPP1 interaction; truncated 5-HT$_{2C}$ R tail proteins missing the terminal PDZ binding motif failed to show detectable interaction with MUPP1. There are four recognized classes of carboxyl-terminal PDZ binding motifs to date (48). The carboxyl terminus of the 5-HT$_{2C}$ R belongs to a type 1 PDZ binding motif (*X*-Ser/Thr-*X*-Val/Ile/Leu-COOH). Ser^{458} of the 5-HT$_{2C}$ R is predicted to be a critical residue for interacting with the PDZ 10 domain backbone. Since both Ser^{458} and Ser^{459} of the 5-HT$_{2C}$ R are phosphorylated upon ligand activation (2), we examined the role of receptor phosphorylation on the 5-HT$_{2C}$ R-MUPP1 interaction.

In the next series of experiments, we investigated whether mutations of the terminal serines to aspartates that mimic phosphorylation of serines would affect binding of the 5-HT$_{2C}$ R to PDZ 10 of MUPP1. S458D and S458D/S459D mutations led to a significant loss of receptor tail interaction to PDZ 10. These data are in agreement with crystal structure data indicating that the hydroxyl group of the Ser at the −2 position of a type I PDZ binding target is important for hydrogen bonding to His at the first position of the α B helix (α B1) of a type I PDZ domain such as PDZ 10 (21). Our data suggest that the introduction of a negatively charged group at the −2 position of the receptor tail disrupts the hydrogen bonding to the PDZ 10 domain resulting in a loss of interaction. The S459D mutation, on the other hand, retains wild-type interaction. This result is also consistent with structural studies showing that the side chain of the −1 position residue of a PDZ binding target does not hydrogen-bond to residues in the PDZ domain backbone but rather points away from the pocket (21). Interestingly, c-Kit, a receptor tyrosine kinase whose carboxyl-terminal sequence ends in Asp-Asp-Val, and claudin-1, a tight junction protein whose carboxyl-terminal sequence ends in Asp-Tyr-Val, have been identified to interact with PDZ 10 of MUPP1 (49, 50). These observations are unexpected in light of structural information on PDZ-ligand complexes, including the current data of carboxyl-terminal mutants in the 5-HT$_{2C}$ receptor. One possible explanation is that c-Kit and claudin-1 interaction with PDZ 10 may involve additional residues upstream from the carboxyl termini.

To determine whether serotonin stimulation within a cellular context would alter 5-HT$_{2C}$ R binding to PDZ 10, we examined the receptor stably expressed in NIH-3T3 cells in the absence or presence of serotonin. A dose-dependent loss of interaction of the receptor with MUPP1 occurred when receptor was incubated with increasing concentrations of serotonin. Furthermore, this concentration curve parallels serotonin-induced receptor phosphorylation, suggesting that a change in receptor phosphorylation state regulates binding to MUPP1 PDZ 10. The effect of serotonin was blocked by the 5-HT$_{2C}$ R antagonist, BOL. Moreover, alkaline phosphatase treatment reversed the effect of serotonin. Interestingly, in the absence of serotonin, phosphatase treatment also resulted in increased receptor binding to PDZ 10. We speculate that this may be indicative of constitutive phosphorylation; basal phosphorylation for this receptor was previously observed by Westphal *et al.* (51) when cells were metabolically labeled with [^{32}P]orthophosphoric acid. Alternatively, an unidentified protein that attenuates MUPP1 binding to the receptor may be reduced by phosphatase treatment and account for this observation. When receptor was stimulated with a maximal serotonin-mediated receptor phosphorylation dose, agonist washout experiments demonstrated that receptor interaction with MUPP1 was restored in a time-dependent manner. We speculate that this restored binding between the receptor and MUPP1 is the result of endogenous phosphatases. Results from antagonist, phosphatase treatments, and agonist washout experiments demonstrated the specificity of our analyses and support the hypothesis that phosphorylation regulates the interaction of 5-HT$_{2C}$ R with MUPP1. A thorough investigation of the kinase responsible for phosphorylation of the 5-HT$_{2C}$ R has been initiated. Initial studies suggest that the usual second messenger-activated kinases are not involved (data not shown). This finding is consistent with investigations of another G-

protein-coupled receptors, the β-adrenergic receptors, which have identified GRK2 and GRK5 as the major kinases responsible for phosphorylating receptor carboxyl threonine and serine residues (40, 52, 53).

Regulation of PDZ-protein interactions by phosphorylation is emerging as an important modulator of cellular signaling. Phosphorylation regulation of a PDZ domain interaction was first observed in the potassium channel Kir 2.3 (37). Protein kinase A phosphorylation of Kir 2.3 causes a disruption of its interaction with PSD-95. Protein kinase A also regulates the interaction between Kir 2.2, another potassium channel, and synapse-associated protein 97, SAP97, resulting in a decreased interaction (38). G protein-coupled receptor kinases have been shown to phosphorylate the β_1- and β_2-adrenergic receptors at carboxyl-terminal serines of a PDZ binding motif, which reduces their interaction with PDZ domain proteins (39, 40, 54). The α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA)glutamate receptor exhibits dual specificity for its interacting partners PICK1 (protein interacting with C kinase) and GRIP/ABP (glutamate receptor-interacting protein)/(AMPA receptor-binding protein), depending upon whether the glutamate receptor 2/3 subunit is phosphorylated by protein kinase C (42–45, 55, 56). Finally, the binding of stargazin, a transmembrane protein that associates with AMPA receptors, to PSD-95 is regulated by protein kinase A phosphorylation (46, 47, 57, 58).

In this paper, we established that phosphorylation of the 5-HT$_{2C}$ R at Ser^{458}, the serine residue critical for binding to PDZ 10, resulted in a decrease in MUPP1 interaction. We also provide evidence that receptor interaction with MUPP1 is sensitive to basal phosphorylation of the receptor. Due to the assembling nature of PDZ proteins, we propose that MUPP1 regulates the coupling of the 5-HT$_{2C}$ R to various effectors to activate downstream signaling processes. MUPP1 interaction with the 5-HT$_{2C}$ R when the receptor is in an unphosphorylated form may keep the receptor in a conformation state (and vice versa) that is masked from some of its downstream signaling partners. Then in an agonist-dependent or -independent manner (an event that is not fully understood), the receptor becomes phosphorylated at the PDZ binding motif, and MUPP1 is released from the receptor, resulting in a change in conformation that reveals downstream signaling molecules scaffolded to MUPP1. Changes in MUPP1 folding have been postulated as a result of observations by Becamel *et al.* (18), showing that when MUPP1 is expressed alone in COS cells, a carboxyl-terminal vesicular stomatitis virus epitope tag is not accessible; however, when MUPP1 is co-expressed with the 5-HT$_{2C}$ R, the tag is accessible. The possibility also exists that the sole function of MUPP1 may be to simply traffic or anchor the 5-HT$_{2C}$ R to specific membrane domains without a direct effect upon receptor signaling. Based upon the data presented here and previous studies on PDZ proteins and receptors, phosphorylation appears to be a critical regulator of PDZ protein-protein interactions, including regulation of the interaction between MUPP1 and the 5-HT$_{2C}$ R. Our results suggest that cells may have an underlying mechanism to dynamically regulate overall cellular activity in the absence and subsequent exposure to agonist by modulating basal receptor phosphorylation and thereby balancing the extent of maximal receptor activation.

Acknowledgments—We thank Drs. Brian Wadzinski and Vsevolod Gurevich for discussions related to phosphatases and kinases. We thank Drs. Mike S. S. Chang and Raymond D. Price for critical review and comments on this manuscript. We also thank Blairanne Williams, Kathleen Rutledge, and Kenyatta Thompson for excellent technical assistance with cell culture.

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Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery

Jeffrey M. Stadel, Shelagh Wilson and Derk J. Bergsma

Access to DNA databases has introduced an exciting new dimension to the way biomedical research is conducted. 'Genomic research' offers tremendous opportunity for accelerating the identification of the cause of disease at the molecular level and thereby foster the discovery of more selective medicines to improve human health and longevity. The current challenge is to close the gap rapidly between gene identification and clinical development of efficacious therapeutics. In the present review, **Jeffrey Stadel, Shelagh Wilson and Derk Bergsma** outline the rationale and describe strategies for converting one large class of novel genes, orphan G protein-coupled receptors (GPCRs), into therapeutic targets. Historically, the superfamily of GPCRs has proven to be among the most successful drug targets and consequently these newly isolated orphan receptors have great potential for pioneer drug discovery.

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The advent of rapid DNA sequencing spawned the 'genomic era', which has led to the initiation of the Human Genome Project. The novel technologies developed in association with genomic research have already had a significant impact on the way investigations into the basis of disease are being conducted and will, no doubt, substantially enhance the means by which diseases are diagnosed and treated in the near future. To keep pace with the evolution of molecular medicine, the pharmaceutical industry has embraced genomics and is attempting to exploit the new technologies to identify novel targets for drug discovery. The major questions that remain to be addressed concern how to convert genomic sequences into therapeutic targets in an expeditious manner and eventually to obtain pharmaceutical drugs that will enhance the quality of life. This review will deal with a single class of novel molecular targets, focusing on the burgeoning collection of G protein-coupled receptors (GPCRs) called 'orphan' receptors[1]. GPCRs are a superfamily of integral plasma membrane proteins involved in a broad array of signalling pathways. Since the first cloning of GPCR gene sequences over a decade ago, novel members of the GPCR superfamily have continued to emerge through cloning activities as well as through bioinformatic analyses of sequence databases, although their ligands are unidentified and their physiological relevance remain to be defined. These 'orphan' receptors provide a rich source of potential targets for drug discovery.

The members of the GPCR superfamily are related both structurally and functionally. The signature motif of these receptors is seven distinct hydrophobic domains, each of which is 20–30 amino acids long and which are linked by hydrophilic amino acid sequences of varied length[2,3]. Biophysical[4] and biochemical[5] studies support the notion that these receptors are intercalated into the plasma membrane with the amino terminus extracellular and the carboxy terminus in the cytoplasmic portion of the cell. Therefore, these receptors are often referred to as seven transmembrane (or 7TM) receptors. While it is not yet known how many individual genes actually encode these receptors, it is clear that this family of proteins is one of the largest yet identified. Functionally, GPCRs share in common the property that upon agonist binding they transmit signals across the plasma membrane through an interaction with heterotrimeric G proteins[6,7]. These receptors respond to a vast range of agents[2,5,8] such as protein hormones, chemokines, peptides, small biogenic amines, lipid-derived messengers, divalent cations (e.g. a Ca^{2+} sensor has been identified that is a GPCR)[9] and even proteases such as thrombin, which activates its receptor by cleaving off a portion of the amino terminus[10]. Finally, these receptors play an important role in sensory perception including vision and smell[2,5,8]. Correlated with the broad range of agents that activate these receptors is their existence in a wide variety of cells and tissue types, indicating that they play roles in a diverse range of physiological processes. It is likely, therefore, that the GPCR superfamily is involved in a variety of pathologies. This point was recently emphasized by the surprising discovery that certain GPCRs for chemokines act as co-factors for HIV infection[11–13].

GPCRs represent the primary mechanism by which cells sense alterations in their external environment and convey that information to the cells' interior. The binding of an agonist to the receptor promotes conformational changes in the cytoplasmic domains that lead to the interaction of the receptor with its cognate G protein(s). Agonist-promoted coupling between receptors and G proteins leads to the activation of intracellular effectors that substantially amplify the production of second messengers feeding into the signalling cascade. Since effectors are often enzymes [e.g. adenylate cyclase[14], which converts ATP to cAMP, or phospholipase C (Ref. 15), which hydrolyses inositol lipids in membranes to release inositol trisphosphate, which in turn mobilizes Ca^{2+} within a cell] or ion channels[16], many second messenger molecules can be produced as the result of a single agonist binding event with its receptor. Changes in the intracellular levels of ions or cAMP, or both,

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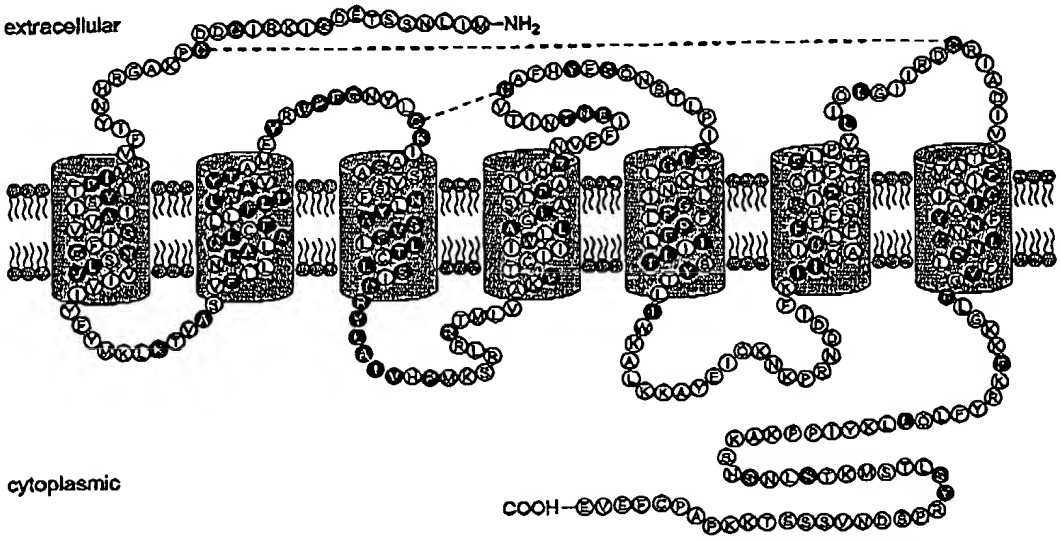

Fig. 1. Comparison of the protein sequence identity of the orphan APJ[19] receptor with the angiotensin AT_1 receptor[20]. The filled circles indicate amino acid identity (29.9%) between the two G protein-coupled receptors (GPCRs). This is a typical example of the protein sequence identity shared between orphan and known GPCRs.

result in the modulation of distinct phosphorylation cascades[17,18], extending through the cytosol to the nucleus, that eventually culminate in the physiological response of the cell to the extracellular stimulus. Although the overall paradigm is apparently the same for all GPCRs, the diversity of receptors, G proteins and effectors suggest a myriad of potential signalling processes and this becomes an important concept as we try to identify the function of orphan GPCRs.

To date, more than 800 GPCRs have actually been cloned from a variety of eukaryotic species, from fungi to humans [see L. F. Kolakowski in GCRDb-WWW The G Protein-Coupled Receptor DataBase World-Wide-Web Site (http://receptor.mgh.harvard.edu/GCRDBHOME.html.org)]. For humans, the most represented species, about 140 GPCRs have been cloned for which the cognate ligands are also known. This number excludes the sensory olfactory receptors, of which hundreds to thousands are predicted to exist. By traditional molecular genetic approaches, coupled with the explosion in genomic information, it has been possible to identify more than 100 additional orphan GPCR family members. By definition, there is enough sequence information in the receptor cDNAs to place them clearly in the superfamily of GPCRs, but often there is insufficient sequence homology with known members of this family to be able to assign their ligands with confidence or predict their function. In total, there are currently over 240 human GPCRs, excluding sensory receptors. As the size of sequence databases continues to increase, this list is expected to grow to 400, and perhaps even to 1000 or more unique gene products. The list will grow even further as paralogues and alternatively spliced GPCR variants emerge. Most orphan GPCRs share a low degree of sequence homology (typically about 25–35% overall amino acid sequence identity), with known GPCRs, suggesting that they belong to new subgroups of receptors (Fig. 1)[19,20]. Indeed, several orphan GPCRs show closer homology to each other than to known GPCRs. Nevertheless, the majority of orphan receptors are phylogenetically distributed among a broad spectrum of distantly related, known receptor subgroups.

What is the rationale for investing considerable time and resources into trying to establish the function of orphan GPCRs? Simply stated, GPCRs have a proven history of being excellent therapeutic targets. Within the past 20 years, several hundred new drugs have been registered that are directed towards activating or antagonizing GPCRs; in fact, it is estimated that most current research within the pharmaceutical industry is focused on this signalling pathway[21]. Table 1 shows a representative snapshot of a variety of receptors, disease targets and corresponding drugs. It is clear from this table that the therapeutic targets span a wide range of disorders and disease states. Another example of the significance and versatility of GPCRs is the number of cases of genetic diseases that are linked to defects in these proteins; some of these diseases are indicated in Table 2 (Refs 22–38). It is likely that many more genetic diseases will be mapped to GPCRs as the era of genomics continues to expand and families with inherited mutations are examined much more comprehensively.

The importance of GPCRs to drug discovery continues to be manifested by the fact that across the pharmaceutical industry active research projects, ranging from basic studies all the way through to advanced development, are focused on GPCRs as primary targets. Molecular biology has had a dramatic influence on these efforts.

Table 1. Examples of marketed drugs for G protein-coupled receptors (GPCRs)

GPCR	Generic	Drug	Indication
Muscarinic acetylcholine	Bethanechol	Urecholine	GI
	Dicyclomine	Bentyl	GI
	Ipratropium	Atrovent	CP
Adrenoceptor			
β_1	Atenolol	Tenormin	CP
α_2	Clonidine	Catapres	CP
β_1/β_2	Propranolol	Inderal	CP
α_1	Terazosin	Hytrin	CP
β_2	Albuterol	Ventolin	CP
$\beta_1/\beta_2/\alpha_1$	Carvedilol	Coreg	CP
Angiotensin			
AT_1	Losartan	Cozaar	CP
	Eprosartan	Teveten	CP
Calcitonin	Calcitonin	Calcimar	Osteoporosis
	eel-Calcitonin	Elcatonin	Osteoporosis
Dopamine			
D_2	Metoclopramide	Reglan	GI
D_2/D_3	Ropinirole	Requip	CNS
D_2	Haloperidol	Haldol	CNS
Gonadotropin-releasing factor	Goserelin	Zoladex	Cancer
	Nafarelin	Synarel	Endometriosis
Histamine			
H_1	Dimenhydrinate	Dramamine	CNS
H_1	Terfenadine	Seldane	CP
H_2	Cimetidine	Tagamet	GI
H_2	Ranitidine	Zantac	GI
Serotonin (5-HT)			
5-HT_{1D}	Sumatriptan	Imitrex	CNS
5-HT_{2A}	Ritanserin	Tisertan	CNS
5-HT_4	Cisapride	Propulsid	GI
5-HT_{1B}	Trazodone	Desyrel	CNS
$5\text{-HT}_{2A/2C}$	Clozapine	Clozaril	CNS
Leukotriene	Pranlukast	Onon	CP
	Zafirlukast	Accolate	CP
Opioid			
κ	Buprenorphine	Buprenex	CNS
	Butorphanol	Stadol	CNS
μ	Alfentanil	Alfenta	CNS
	Morphine	Kadian	CNS
Oxytocin		Syntocinon	Labour
Prostaglandin	Epoprostenol	Flolan	CP
	Misoprostol	Cytotec	GI
Somatostatin	Octreotide	Sandostatin	Cancer
Vasopressin	Desmopressin		CP/Renal

CP, cardiopulmonary system; GI, gastrointestinal system.

Table 2. Diseases associated with mutations of G protein-coupled receptors (GPCRs)

GPCR	Mutation	Disease	Refs
Rhodopsin	Missense: Pro23 to His (NT) Missense: Val87 to Asp (2TM) Missense: Tyr178 to Cys (2EL) Nonsense: Gln344 to Stop (CT)	Retinitis pigmentosa	22, 23
Thyroid stimulating hormone	Missense: Asp619 to Gly (3IL) Missense: Ala623 to Ile (3IL)	Hyperfunctioning thyroid adenomas	24
Luteinizing hormone	Missense: Asp578 to Gly (6TM)	Precocious puberty	25
Vasopressin V_2	Missense: Arg137 to His (2IL) Missense: Gly185 to Cys (2EL) Frameshift at Arg230 (3TM)	X-linked nephrogenic diabetes	26–28
Ca^{2+}	Missense: Arg186 to Glu (NT) Missense: Glu298 to Lys (NT) Missense: Arg796 to Trp (3IL) Missense: Glu128 to Ala (NT)	Hyperparathyroidism, hypocalciuric hypercalcaemia	29, 30
Parathyroid hormone (PTH type b)	Missense: His223 to Arg (1IL)	Short-limbed dwarfism	31
β_3-Adrenoceptor	Missense: Trp64 to Arg (1IL)	Obesity, NIDDM	32–34
Growth-hormone-releasing hormone	Nonsense: Glu72 to Stop (NT)	Dwarfism	35
Adrenocorticotropin	Missense: Ser74 to Ile (2TM)	Glucocorticoid deficiency	36
Glucagon	Missense: Gly40 to Ser (NT)	Diabetes, hypertension	37, 38

Abbreviations: CT, carboxyl terminus; EL, extracellular loop; IL, intracellular loop; NIDDM, non-insulin-dependent diabetes mellitus; NT, amino terminus; TM, transmembrane segment.

The cloning of cDNAs for well-known GPCRs led to the discovery of a surprising number of paralogues[3]. The existence of these novel receptor subtypes was unexpected because the current cornucopia of pharmacological agents does not possess the required selectivity to distinguish all of them clearly, and thus an opportunity for drug discovery was quickly recognized. Current research efforts seek to define the physiology associated with these novel receptor subtypes and to discover highly selective compounds as potential pharmaceutical drugs. These efforts are almost exclusively focused on GPCRs for which activating ligands are known. Since characterized GPCRs were, and continue to be, attractive therapeutic targets, it is most reasonable to speculate that many of the orphan receptors have similar potential. The initial challenge is to determine the function of each orphan receptor through the identification of activating ligands and, once the function is clarified, link the orphan receptor to a specific disease and thus establish it as a candidate for a comprehensive drug discovery effort.

Reverse molecular pharmacology

Until recently, research into the identification of GPCRs as targets for drug discovery has been conducted using the traditional approach illustrated in Fig. 2. For this strategy, the starting point is functional activity, which forms the basis of an assay by which a ligand is identified through purification from biological fluids, cell supernatants or tissue extracts. One example of the success of this strategy is the discovery of the potent vasoconstricting peptide endothelin[39]. Once isolated, the ligand is used to characterize its cellular and tissue biology as well as its pathophysiological role. Subsequently, cDNAs encoding corresponding receptors are 'fished' from gene libraries using a variety of methodologies (e.g. receptor purification and expression cloning) that often either directly or indirectly use the ligand as the 'hook'. As the nucleotide sequences for GPCRs begin to accumulate and be analysed, additional receptors can be cloned by homology screening, by positional cloning, and by polymerase chain reaction (PCR) methodologies that use oligonucleotide primers based on nucleotide sequences conserved within the seven transmembrane domains of the GPCR family. Once the cloned human receptor cDNA is expressed in a heterologous cell system[40], it is used, together with its ligand, to form the basis of a screen to explore chemical compound libraries for receptor antagonists or agonists. Lead structures identified in the screen are refined through medicinal chemistry using an iterative process. Resulting drug leads with appropriate *in vivo* pharmacology are passed on into the clinic for development.

Recently, this paradigm has changed radically with the introduction of a new reverse molecular pharmacological

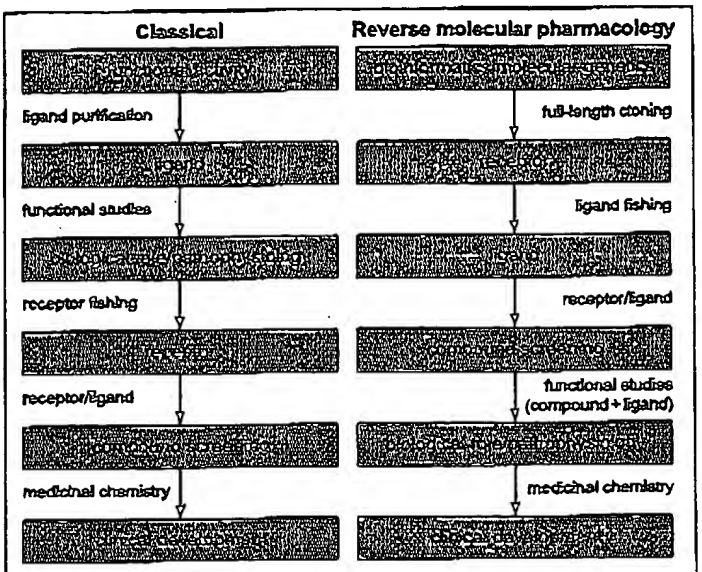

Fig. 2. Paradigm shift from classical to reverse molecular pharmacological approaches for drug discovery.

strategy, shown diagramatically in Fig. 2. Through both traditional molecular cloning techniques and, more recently, mass sequencing of expressed sequence tags (ESTs) from cDNA libraries, it is now possible to identify GPCRs through computational or bioinformatic methodologies. The EST approach, initially proposed by Sidney Brenner (University of Cambridge) and first brought to large-scale practice by Craig Venter (The Institute of Genome Research), constitutes random, single-pass sequencing of cDNAs randomly picked from a collection of cDNA libraries, followed by extensive bioinformatic analysis of the sequence to identify structural signatures characteristic of GPCRs. Once new members of the GPCR superfamily are identified, the recombinantly expressed receptors are used in functional assays to search for the associated novel ligands. The receptor–ligand pair are then used for compound bank screening to identify a lead compound that, together with the activating ligand, is used for biological and pathophysiological studies to determine the function and potential therapeutic value of a receptor antagonist (or agonist) in ameliorating a disease process. In addition, clues as to therapeutic potential may involve receptor genotyping of disease populations. Once a link with a disease is finally identified, an appropriate compound can be advanced for clinical study.

The reverse molecular pharmacological strategy is a far more daunting challenge and risky endeavour when compared with the more traditional approach, since the starting material for a drug discovery effort is simply an orphan receptor of unknown function, with no apparent relationship to a disease indication. However, the potential reward of using this approach is that resultant drugs naturally will be pioneer or innovative discoveries, and a significant proportion of these unique drugs may be useful to treat diseases for which existing therapies are lacking or insufficient.

Screening strategy

Figure 3 illustrates the generic strategy that we use for our reverse molecular pharmacological approach. In addition to the EST approach, which has yielded the majority of our collection of orphan receptors, we have also used a number of more traditional approaches such as low-stringency screening, using portions of known GPCRs as hybridization probes, as well as PCR-based methods. By these techniques we have succeeded in identifying more than 70 orphan receptors in addition to those already cited in the literature.

Since cDNAs identified by EST cloning are often incomplete, northern hybridization analysis is used to establish the tissue or cell pattern of mRNA expression of the GPCRs. This information is used to identify the tissue or cell cDNA libraries that are to be probed for full-length clones and, significantly, to determine whether a receptor is expressed in a particular normal or diseased tissue of interest. A highly selective tissue expression pattern may also provide a clue with respect to receptor function. Once obtained, full-length GPCR clones are expressed in mammalian cell lines and yeast model systems (see below) for functional analysis. *Xenopus* oocytes may also be used for expression; however, low screening throughput limits their use to a secondary, confirmatory assay system. For mammalian cell expression, the human embryonic kidney (HEK) 293 cell line or Chinese hamster ovary (CHO) cells are frequently used. These cell types possess a large repertoire of G proteins that are necessary for coupling to downstream effectors *in situ*. They also share a reliable history of positive functional coupling for a wide variety of known GPCRs. However, since receptor coupling cannot be accurately predicted from primary sequence data, orphan GPCRs may need to be expressed in a variety of cell lines to establish viable coupling.

These heterologous expression systems form the basis for screening for an activating ligand. The success of establishing functional coupling of the recombinant receptor depends to a large extent on whether the receptor is properly expressed, which may be assessed by northern or Western blot analysis, and whether appropriate G proteins and downstream effectors are present in the cell in which the receptor is expressed. There are several major technical challenges to be met in order to initiate ligand fishing. Because it is difficult to predict accurately the coupling specificity of orphan GPCRs from their primary sequence, assays must be chosen that will detect a wide range of coupling mechanisms. These generally focus on changes in intracellular levels of cAMP or Ca^{2+} but can also include more generic measurements, such as metabolic activation of the cell via the cytosensor microphysiometer[n]. Recently, it has become possible to implement most of these screens in high-throughput format by using fluorescent-based

assays and using charge-coupled device cameras and reporter gene constructs that allow easy readout of the assay on microtitre plates. Ever increasing throughput of the assays will be necessary to screen large libraries. However, this approach is somewhat cumbersome and inefficient if all the assays described above have to be used. Is it possible to funnel heterologous signal transduction through a defined pathway? The prospect of an assay for a single transduction pathway comes from the observation that heterologous expression of the G protein subunit $G_{\alpha 15/16}$ promoted coupling of various GPCR subfamily members through activation of phospholipase Cβ and likely Ca^{2+} mobilization[42,43]. Although this approach may not work universally, the diversity of the GPCRs successfully coupled through $G_{\alpha 16}$ to phospholipid metabolism suggests that this could be a useful method to screen for orphan receptor activation.

Once heterologous receptor expression is achieved and functional assays are in place, ligand fishing experiments can be initiated. Although the homology with known GPCRs is low, we nevertheless begin by screening the orphans against known GPCR ligands; since the sequence homology between some subtypes of known receptors can be low (e.g. 30–40% between neuropeptide Y receptor subtypes), it is possible that new paralogue receptors for known ligands still remain to be discovered. The next step is to search for novel activating ligands by screening biological extracts obtained from tissues, biological fluids and cell supernatants. An additional option is screening libraries of compounds for activating ligands. Complex libraries of peptides or compound collections could be rich sources of 'surrogate' agonists that would promote receptor activation and coupling but are not endogenous ligands. The rationale for searching for surrogate agonists springs from a report that a nonpeptide agonist has been discovered for the angiotensin II receptor[44]. There is also an obvious precedent for nonpeptide agonists for opioid receptors. Screening of the very large libraries that will be generated by fractionation of biological extracts and by combinatorial chemical synthesis requires that the functional assays used have not only a high throughput but are also robust, since false positives can be a significant problem.

Examples are beginning to emerge from several efforts showing that progress has been made in characterizing orphan GPCRs. A first example is the identification of an orphan GPCR that functions as a calcitonin gene-related peptide (CGRP) receptor[45]. CGRP is a peptide of 37 amino acids, widely distributed in neurones, and functions as a potent vasodilator. It may be involved in migraine and has been implicated in non-insulin-dependent diabetes mellitus because it promotes resistance to insulin. An orphan GPCR EST was derived from a human synovium cDNA library[45]. Sequence analysis showed that the new GPCR has ~56% similarity to the human calcitonin receptor and was hence originally expected to be a new subtype of the calcitonin receptor. The message for this novel receptor was expressed predominantly in lung, which is known to be a relatively rich source of CGRP receptors. Following full-length cloning from a human lung library, the orphan receptor cDNA was stably expressed in HEK293 cells. Both radioligand binding using ^{125}[I]CGRP, as well as functional assays of CGRP-stimulated cAMP accumulation, demonstrated an appropriate pharmacological profile for the expressed receptor similar to that observed with endogenous CGRP receptors on human neuroblastoma cells. In addition to identifying the CGRP receptor, the reverse molecular pharmacology approach has also been used to identify other orphan receptors, such as the anaphylatoxin C3a receptor[46].

The examples given above are for receptors with significant homology to known GPCR superfamily members and their activating ligands proved to be known GPCR ligands. Will ligand fishing be successful in identifying novel endogenous ligands? Recently, two groups

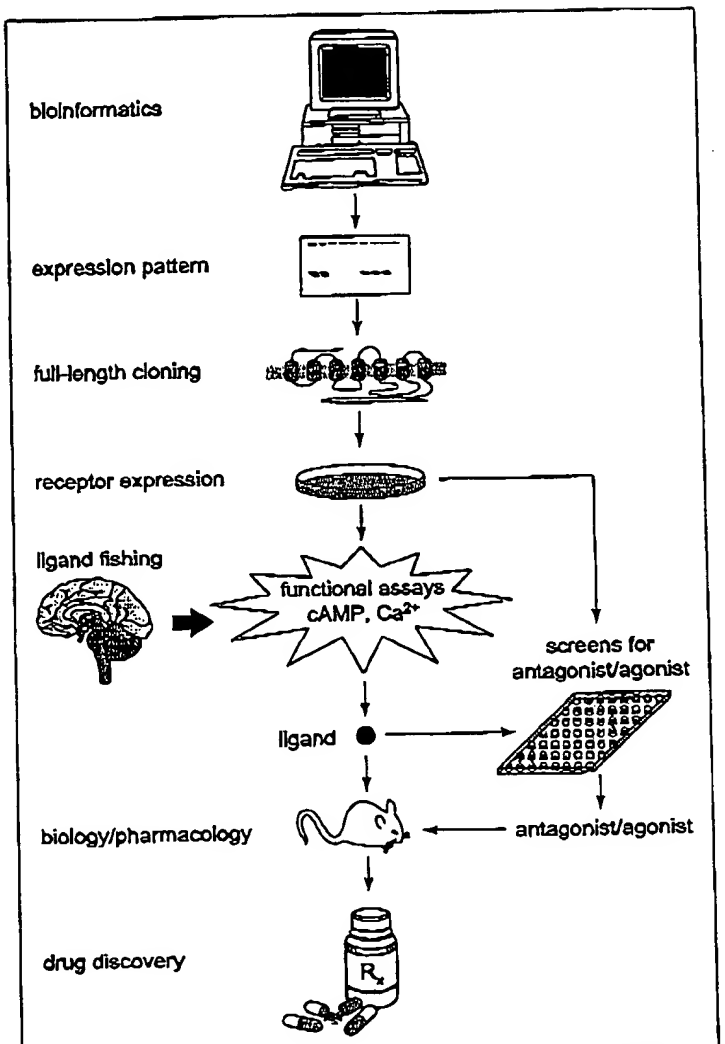

Fig. 3. Strategy for using orphan G protein-coupled receptors as targets for drug discovery.

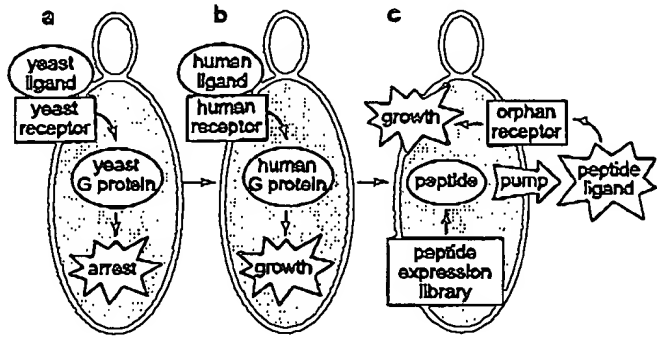

Fig. 4. Yeast-based screen for the identification of agonists for orphan G protein-coupled receptors (GPCRs). a: Normal, endogenous GPCR signalling in yeast (*Saccharomyces cerevisiae*). b: Substitution of a human GPCR and a human G_α subunit for yeast counterparts and modification of downstream signalling pathways such that agonist stimulation of the recombinant GPCR promotes growth. This yeast strain can be screened using biological extracts or compound libraries, or both. c: Yeast cells can be engineered to secrete small peptides from a random peptide library to identify autocrine surrogate, peptide agonists for recombinant orphan GPCRs. Modified from Ref. 49, with permission.

investigated an orphan opioid-like receptor, ORL1 (Refs 47 and 48). Both groups expressed the orphan GPCR in CHO cells and challenged the transfected cells with a series of opiate agonists, but without response. Both groups then used a similar ligand fishing approach. Taking crude extracts from rat brain[47] or porcine brain[48], they screened against the stably transfected cell lines using inhibition of adenylate cyclase activity as a functional assay. They were able to fractionate the brain extracts and identify the novel dynorphin-like ligand, which they called nociceptin[47] or orphanin FQ (Ref. 48). Thus, both teams successfully established a functional assay in transfected CHO cells that allowed the purification of a novel neuropeptide ligand that is 17 amino acids long for the orphan receptor. This work validates the ligand fishing approach for characterizing the function of orphan GPCRs.

Concluding remarks and future challenges

Although orphan GPCRs have been around for over ten years, very few companies have, until recently, been willing to risk their resources to explore opportunities among this category of receptors. However, the environment for the pharmaceutical industry has changed due to the confluence of several major technological advances. The conversion of gene sequences encoding GPCRs to drug targets is substantially aided by the development of combinatorial chemistry methods and miniaturized high-throughput screening techniques. The future challenge for drug discovery in this arena is to integrate these technologies innovatively and productively. One glimpse of the future comes from the field of functional genomics. The endogenous GPCR transduction system of the yeast, *Saccharomyces cerevisiae*, which is the pheromone pathway required for conjugation and mating, has been commandeered – through genetic engineering – to permit functional expression and coupling of human GPCRs and humanized G protein subunits to the endogenous signalling machinery[49–51] (Fig. 4). Further manipulations involve conversion of the normal yeast response to pheromone or activating ligand (growth arrest) to positive growth on selective media or to reporter gene expression. In addition, yeast cells have been engineered to express and secrete small peptides from a random peptide library that will permit the autocrine activation of heterologously expressed human GPCRs (Refs 49 and 51). This provides an elegant means of screening rapidly for surrogate peptide agonists that activate orphan receptors. This yeast system is, of course, not limited to autocrine ligand screening but can also be used in high-throughput mode to screen directly the fractions from biological extracts and the various chemical libraries as described above. A major advantage of the yeast system over the mammalian heterologous expression systems is its ease of use and its lack of endogenous GPCRs, which can confound ligand fishing expeditions in mammalian cells.

There is now tremendous pressure to be the first on the market with highly selective drugs that target therapeutic areas of unmet medical need and ideally have novel mechanisms of action. As a consequence, the pharmaceutical industry has recognized the power of genomics to provide it with new and unique drug targets. Genomics has responded with a plethora of novel proteins, included among them over 100 orphan GPCRs. Because of the proven link of GPCRs to a wide variety of diseases and the historical success of drugs that target GPCRs, we believe that these orphan receptors are among the best targets of the genomic era to advance into the drug discovery process.

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